Morphology and Diversity of Arbuscular Mycorrhizal Fungi Colonizing Roots of Dandelion and Chive

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ABSTRACT

Arbuscular mycorrhizas (AM) are the plant root-fungus interactions that are most widespread mycorrhiza in nature. As classically defined, there are two major AM morphologies named after the plant genera in which they were first described: *Arum*- (intercellular hyphae with arbuscules mainly in inner root cortex), *Paris*- (extensive hyphal coils in outer root cortex), as well as intermediate morphotypes. In this study, dandelions and chives harvested in Saskatoon (SK, Canada) were examined for AM colonization and morphological types. A Multiple Quantitation Method (MQM) was used for assessing fungal colonization intensity using magnified epifluorescence images of lactofuchsin stained roots, plus details analyzed by high-resolution confocal fluorescence imaging. The results showed that host plants harbored diverse endorhizal fungi, including arbuscular mycorrhizal fungi (AMF), septate endophytes (SE) and fine endophytes (FE), with varying abundances. The soil properties were assessed with respect to P status, organic matter and pH, but there was no correlation with the fungal abundance in this study. Both dandelion and chive roots had *Arum*- and *Paris*-type AM. In order to assess the applicability of a current model, I studied quantitative relationship between the cell packing pattern and AM morphotype. Cross sections of host roots were analyzed with Image J software to calculate the proportion of air spaces. The abundance of arbuscules (*Arum*-type) and hyphal coils (*Paris*-type) were significantly different in chive and dandelion roots. However, there was no difference in the proportion of air spaces in the inner or outer cortex. Therefore, host root cell packing does not appear to influence AM morphotype at least in the samples in this study. AM fungal diversity was preliminarily investigated by nested PCR with group specific primers, showing multiple PCR bands within root samples, and indicating the potential complexity of AMF groups. Further work to sequence the PCR products is needed to elucidate the AMF groups present.

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LIST OF ABBREVIATIONS

AM ..... Arbuscular mycorrhiza
AMF ..... Arbuscular mycorrhizal fungi
Amp ..... Ampicillin
ANOVA ..... Analysis of Variance
AS ..... Air-space
BSA ..... Bovine serum albumin
CBE ..... Chlorazole Black E
CLSM ..... Confocal laser scanning microscopy
DDSA ..... Dodecenyl succinic anhydride
DLAG ..... 1:1:1 distilled water: 85 % lactic acid: glycerol
DMP-30 ..... Tri (dimethylaminomethyl) phenol
FE ..... Fine endophyte
Glc ..... Glucose
ITS ..... Internal Transcribed Spacer
LB ..... Luria Broth
LF ..... Lactofuchsin
LSU ..... Large subunit
MQM ..... Multiple Quantitation Method
MYA ..... Million years ago
NMA ..... Nadic methyl anhydride
NMR ..... Nuclear magnetic resonance
P ..... Phosphorus
Pi ..... Inorganic phosphorus
PCR ..... Polymerase Chain Reaction
PVPP ..... Polyvinylpolypyrrolidone
rDNA ..... Ribosomal DNA
rRNA ..... Ribosomal RNA
Ri T-DNA ..... Root-inducing transferred-DNA
SE ..... Septate endophyte
SSU ..... Small subunit
TEM ..... Transmission electron microscopy

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I. Introduction

1.1 Mycorrhiza: Definition and types

A mycorrhiza (from the Greek for *fungus roots*; *mycorrhizae* or *mycorrhizas* as plural form) is a mutually beneficial association between the fungus and the roots of a plant. Mycorrhizas benefit the survival of most plants (Leung et al. 2006; van der Heijden et al. 2006) and are ubiquitous across terrestrial ecosystems. Mycorrhiza associations have been classified into seven groups according to morphological and histological criteria. Arbuscular mycorrhizas (AM) and ectomycorrhizas (ecto) are the most prevalent types, relative to the arbutoid, monotropoid, ectendo, ericoid and orchid mycorrhizas which can only be found in a few plant species (Smith and Read 1997). Ectomycorrhizas normally form between the roots of woody plants, particularly in the families Pinaceae and Cupressaceae, and fungi belonging to the phyla Basidiomycota and Ascomycota. AM are present in most habitats and are defined as the association between fungi of the phylum Glomeromycota and about 80% of terrestrial plant species (Trappe 1987).

1.2. Arbuscular mycorrhiza (AM) symbiosis

1.2.1. History

Kidston and Lang (1921, reviewed in Trappe 1987) first reported AM fossils found in Rhynie, Aberdeenshire, Scotland that dated back to the Devonian (395-345 million years ago, MYA). Carboniferous fossils (345-280 MYA) of fern roots revealed non-septate hyphae and arbuscule structures, which indicated rhizome colonization (Remy et al. 1994). Redecker et al. (2000b) discovered fossil spores and hyphae consistent with glomalean fungi in 460 million-year-old rocks from the Ordovician. In combination with previous fungal phylogeny analysis from fossil records, the major groups of terrestrial fungi likely originated before 600 MYA (Redecker et al. 2000b). AM associations may have assisted ancestral vascular plants to invade land

(Pirozynski and Malloch 1975) and this symbiotic association has likely been a major influence in the evolution of terrestrial ecosystems.

AM fungi (AMF) are members of the order Glomales, class Glomeromycetes in the new phylum Glomeromycota (Schussler et al. 2001). There are eight recognized genera of AMF: *Glomus, Paraglomus, Sclerocystis, Scutellospora, Gigaspora, Acaulospora, Archaeospora* and *Entrophospora*, including approximately 150 species (Peterson et al. 2004). However, species delineation remains in a state of revision with the application of molecular techniques (Morton and Redecker 2001).

1.2.2. Habitat

AM are found in almost all terrestrial habitats. AM are of high diversity in plant communities such as tropical rainforests (Husband et al. 2002; Janos 1980) and temperate grasslands (Anderson et al. 1984), whereas the nutrient-rich agricultural soils display a low AM diversity. Soil disturbances in agricultural soils, such as tillage, fertilizer and crop rotation, could disrupt the hyphal growth (Jasper et al. 1991; Kabir et al. 1998) and cause excess P/Zn (Thompson 1987, 1994). But the agricultural practices are not always synergistic with the mycorrhiza symbiosis whether a high-input/conventional or low-input/organic system is employed (Ryan and Graham 2002). AM populations are lower in very arid soils, such as dunes (Koske 1987) and deserts (Virginia 1986). AM are also found in aquatic (Smith and Read 2002) and semi-aquatic grass habitats (Miller 2000).

1.2.3. Carbohydrate supply

AM are dependent on the host for their carbon nutrition in the form of the hexoses glucose (monosaccharide $C_6H_{12}O_6$), fructose (an isomer of glucose), and sucrose (disaccharide $C_{12}H_{22}O_{11}$) (Harley and Smith 1983). The plant may put up to 20% of its carbon investment (Pfeffer et al. 1999) in the mycorrhizal network in order to be

efficiently supplied with phosphorus. Although the fine details of molecular mechanisms of carbon transfer are still largely unknown, the transfer of carbon from plant to fungus may occur through the arbuscules or intraradical hyphae (Pfeffer et al. 1999), as there is high ATPase activity in the membranes, showing a capacity for active transport (Gianinazzi et al. 1991). Studies using stable isotopic labeling and nuclear magnetic resonance (NMR) spectroscopy provide further evidence of carbon metabolic activities in the symbiosis. Carbon taken up from the host in the form of hexose is converted to trehalose (an alpha 1,1 disaccharide synthesized by fungi) and glycogen (a branched polyma of glucose, α1-4 and α1-6), typical fungal carbohydrates (Shachar-Hill et al. 1995). *In vivo* microscopic observations indicate that some of these storage fungal carbohydrates are transported from the intraradical mycelium to the extraradical mycelium (Pfeffer et al. 1999).

1.2.4. AM benefit: phosphorus (P) uptake and transport

The benefit of mycorrhizae to plants is mainly attributed to an increased uptake of nutrients, especially phosphorus (P), an important plant macronutrient building essential molecules such as nucleic acids and phospholipids and regulating enzymatic and metabolic reactions (Theodorou and Plaxton 1993). The form of P most readily accessed by plants is inorganic P (Pi) (Bieleski 1973). Free Pi levels in soil solutions usually range from less than 1 μM to 10 μM (Marschner and Marschner 1995). The form of Pi existing in soil solution changes with pH. Plants reach highest Pi uptake rates between pH 5.0 and 6.0, where $H_2PO_4^-$ dominates (Ullrich-Eberius et al. 1984; Furihata et al. 1992).

Plants capture P either at the soil-root interface through the root epidermis and root hairs or via external AM hyphae in soil (Smith et al. 2003). However, AMF can be much more efficient than plant roots in capturing P. Cox and Tinker (1976) measured the quantity of P transferred per unit time and divided it by the area of the interface (measuring mosaics of electron micrographs from fixed transverse sections) to

estimate the P flux at 13 nmol/m^2/s^1 along the hyphae in mycorrhizae, which is up to six times faster than that of the root hairs (Bolan 1991). The extraradical hyphae growing into the surrounding soil also increase the surface area of soil contact. In addition, available P concentration in the soil can be increased by mycorrhizal activity, as mycorrhizae lower the rhizosphere pH due to selective uptake of NH_4^+ and release of H^+ ions. Lower soil pH increases the solubility of P compounds (Hamel 2004).

An increase in carbon influx from the plant to the AMF has been positively correlated with the increase in the uptake and transfer of phosphorus from fungi to plant (Bucking and Shachar-Hill 2005). Bucking and Shachar-Hill (2005) used ^{14}C-labelled carbohydrates, ^{33}P-phosphate, and energy dispersive X-ray micro-analysis to follow the uptake and transfer of carbon and P in axenic-cultured transformed carrot roots inoculated with AMF. Species of AMF differ in their abilities to supply the plant with P. In some cases, the role of P uptake can be completely taken over by the AM: Smith et al. (2003) inoculated AMF species to different host plants found that 100% of P was delivered to flax via the mycorrhizal pathway once innoculated with *Glomus intraradices*. However, *Gigaspora rosea* poorly colonized all three plants studied (flax, medic and tomato) and it delivered much less P, indicated by growth depression of host plants.

1.2.5. Plants involved in AM symbiosis

1.2.5.1. Host plants

A wide range of plant species (> 200,000; Fitter and Moyersoen 1996) is found to host AMF and to play important roles in mycorrhizal development, spore formation, and fungal distribution. Helgason et al. (1998) found that spore development of *Glomus fasciculatum* was significantly influenced by the host plant, but not so for *Glomus mosseae* and *Glomus macrocarpum*. Al-Raddad (1993) and Sieverding (1990) also suggested that hosting of certain AMF might be influenced by the variety of plant

species in natural ecosystems.

1.2.5.2. Root anatomy

There are two major categories of root system. The taproot system of dicots is characterised as a single dominant taproot with fibrous lateral roots running outwards, whereas the diffuse root system of monocots is featured for the fibrous and branched roots almost without primary root (Fig.1.2.5.2.1).

Fig.1.2.5.2.1 Diagram of taproot (a) and diffuse root (b) systems.

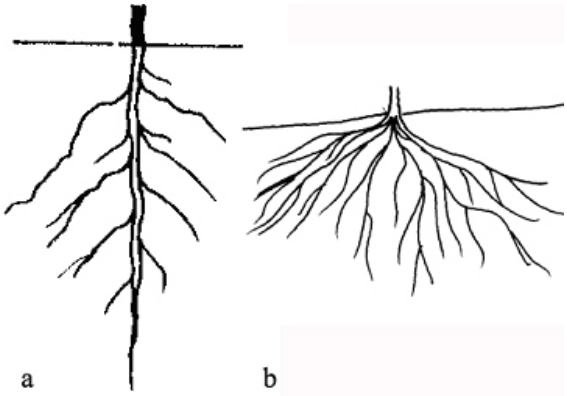
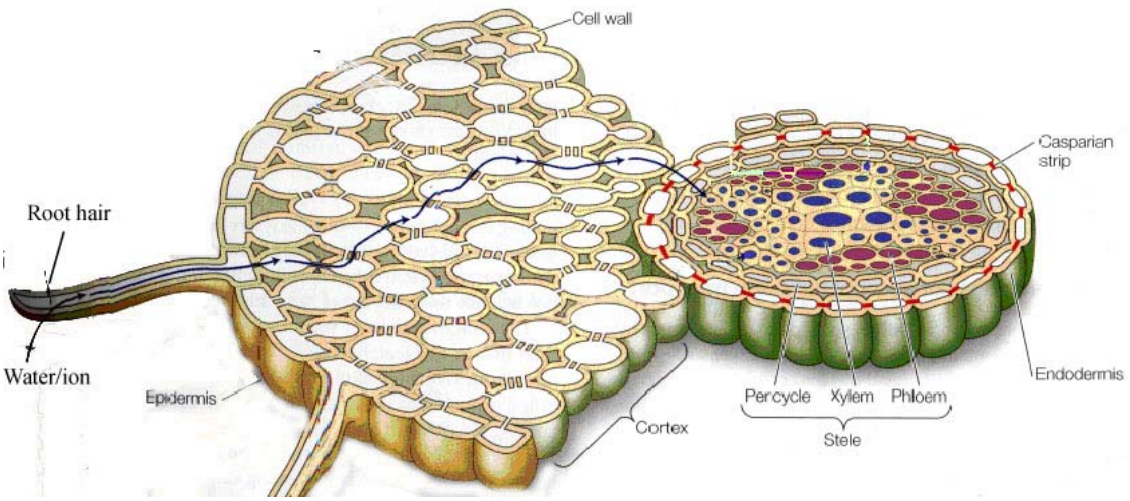


Fig.1.2.5.2.2 Diagram of root structure, showing root hair, epidermis, cell wall, cortex, xylem, phloem, pericycle (stele), endodermis, Casparian strip and the paths of water and ion from root hairs into stele (Sadava et al. 2006).



The outer cell layer of the root is the epidermis (Fig.1.2.5.2.2). The epidermal cells absorb water and produce outgrowths called root hairs, which also take in water and nutrients. Root hairs are very delicate and generally short-lived (a few days) and are continually replaced by newly formed root hairs. Beneath the epidermis is the cortex, which comprises the bulk of the primary root. Within the cortex are intercellular air spaces, that are essential for the aeration of the root. Without these spaces, the cells of the root would be deprived of oxygen and die. The endodermis is a thin layer of small cells forming the innermost part of the cortex and surrounding the vascular tissues deeper in the root. The vascular cylinder consists of the cells interior to the endodermis and contrains xylem tissue that conduct water. AMF never penetrate the endodermis to the vascular cylinder (Reinhardt 2007).

1.2.6. Soil environment

Soil is a complex mixture of living (bacteria, fungi, animals) and nonliving components, including finely ground rock particles of varied size. Sandy soil is very porous, with large spaces between soil particles and while it can absorb much water, little is retained and it dries out quickly. Loam soil is loose, porous and holds water well. Organic materials are important components of loam soil. Clay soil is dense with few air spaces and holds water so tightly that little is available for plants. Soil particles can be aggregated together to different sizes (microaggregates smaller than 250 μm; macroaggregates larger than 250 μm). Soil aggregates are important in helping store organic carbon by protecting them entrapped in the aggregates (Jastrow 1996; Six et al. 2000).

1.2.7. Development of AM symbiosis

1.2.7.1. Presymbiosis

The development of AMF prior to root colonization consists of three stages: spore germination; hyphal growth; and appressorium formation. Spores can lay dormant for months or years until the proper conditions for fungal development are available, such as soil property, temperature, carbon dioxide concentration, pH and P concentration (Douds and Nagahashi 2000). The germ tube will elongate until total consumption of the spore reserves (Helene and Corbiere 2002). Root exudates, including small molecules (sugars, amino acids, organic acids and amides), released from potential mycorrhizal plant roots act as a stimulator for AMF germination (Becard and Piché 1989; Elias and Safir 1987; Giovannetti et al. 1993), whereas exudates from roots of non-mycorrhizal species have no effect on germination (Becard and Piché 1990; Gianinazzi 1989; Giovannetti et al. 1994) or appear to be inhibitory (Koide and Schreiner 1992; Vierheilig and Piché 1996). In natural soils, AM colonization may be initiated from infected root segments with protruding external hyphae as well as a germinating spore (Marsh and Schultze 2001).

The growth of extraradical hyphae in the soil is influenced by mycorrhizal root exudates and soil P concentration (Nagahashi et al. 1996). Fungus-root contact often occurs at the emerging lateral roots where the primary root walls break. Cell wall fragment is a type of root exudate. Studies by Nagahashi et al. (1996) examined the effect of P concentration upon growth of pregerminated spores and extraradical hyphae of *Gigaspora margarita. In vitro,* 1 mM P significantly decreased branching of the primary germ tube, whereas 10 mM P inhibited both hyphal growth and branching. In addition, germinated spores exposed to exudates produced by Root-inducing transferred-DNA (Ri T-DNA) of *Daucus carota* L. grown in the presence of P showed significantly less hyphal branching than those exposed to exudates produced by P-stressed roots. Therefore, low soil P concentrations can increase hyphal growth and branching as well as induce plant exudation.

Extraradical hyphae can produce swellings between epidermal cells. These swellings, termed appressoria, are the sites where hyphae penetrate into the root cortex (Gianinazzi 1996).

1.2.7.2. Penetration and hyphal growth

AM hyphae penetrate root hair cells or epidermal cells by exerting a hydrostatic pressure with the hyphal tip and by producing localized cell wall degrading enzymes (Bonfante and Perotto 1995). Studies on *Glomus mosseae* showed higher activities of cell wall degrading enzymes, pectinase and cellulase in AM colonized roots than non-mycorrhizal roots (Garcia-Garrido et al. 1992; Garcia-Romera et al. 1991).

After AM penetration into a receptive root, aseptate hyphae form a colony by growing through the cortex in both directions from the entry point. The host response to intracellular hyphal invasion (ie. arbuscules or hyphal coil formation) includes forming a host-derived membrane, which is related to and continuous with the plant

cell membrane (Bonfante and Perotto 1995).

1.2.7.3. Arbuscules

Arbuscules are formed by repeated branching of intracellular hyphae. They were named as such by Gallaud (1905), because they resemble little trees. They start from a trunk hypha (5-10 μm in diameter) eventually proliferating to form fine branch hyphae (< 1 μm diameter). The arbuscules create an invagination of the host plasma membrane, increasing the contact area between fungus and cortical cells. They remain outside the cytoplasm of the host cell separated by a periarbuscular membrane, a new subcellular symbiotic compartment (Genre and Bonfante 1997), where carbon and phosphate transfer between two partners (Smith and Smith 1990). The formation of arbuscules is accompanied by plant vacuole fragmentation and the movement of the nucleus to the center of the cell (Bonfante and Perotto 1995). Each arbuscule will form, collapse and degenerate within around 7 to12 days once their activity of nutrient exchange ends, leaving the plant cell undamaged and capable of hosting another arbuscule (Alexander et al. 1989).

1.2.7.4. Vesicles

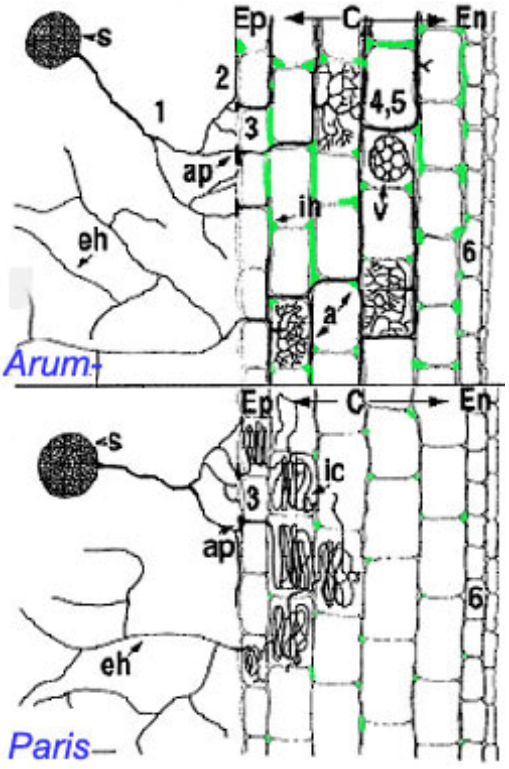
Vesicles are hyphal swellings in the root cortex that contain lipids and nuclei (Smith and Smith 1997) and are thought to act as propagules, which means hyphae containing vesicles can be used to inoculate previously uninfected roots (Biermann and Linderman 1983; Declerck et al. 1998). Vesicles are not present in every AM symbiosis, and some AMF even do not develop them at all (Smith and Smith 1997). In some fungal species, vesicles can be inter- or intracellular, and can remain viable in roots for months or years. Peterson et al. (2004) found that vesicles started to grow soon after the arbuscules and continued to develop when the arbuscules senesced.

1.2.8. AM morphotypes

1.2.8.1. *Arum*-, *Paris*-, and intermediate morphotypes

Two major AM morphotypes, the *Arum*- and *Paris*-types, were named after the plant species in which they were first described *Arum maculatum* and *Paris quadrifolia* (Gallaud 1905). Both AM morphotypes start colonizing host roots with root epidermal penetration, which gives rise to coiled intracellular hyphae in the hypodermis or outer cortex (Allen et al. 2006). In *Arum*-type AM, the intracellular hyphae are limited, whereas in *Paris*-type AM they are extensive.

Fig.1.2.8.1. Cartoon illustration of AM morphotypes (modified after Barker et al. 1998). Ep, Epidermis; C, cortex; En, endodermis; s, spore; eh, external hypha; ap, appressorium; ih, intercellular hypha; a, arbuscule; ic, intracellular coil; and v, vesicle. The air space (AS) is marked as green color.



Arum-type AM (Fig.1.2.8.1 A) have extensive intercellular hyphae with highly branched intracellular arbuscules growing in the cortex. There are reports showing that arbuscules preferentially form in the inner cortex (Abbott 1982; Fisher and Jayachandran 1999; Resendes et al. 2001; Harrison 2005; Allen et al. 2006), where inner and outer cortex were defined as the two cortical cell layers adjacent to the stele and epidermis, respectively (Cavagnaro et al. 2001b). Arbuscule production in cells of the inner cortex was noted in *Trifolium subterranean* inoculated with a range of *Glomus* species (Abbott 1982). In *Medicago truncatula* root colonized with *Glomus intraradices,* the AM fungus grows intercellularly through the outer cortex and forms arbuscules within the inner cortex (Harrison 2005). Fisher and Jayachandran (1999) also showed that *Serenoa repens* (Bartr.) Small, a palm native to the southeastern USA, displayed *Arum*-type AM, showing intercellular hyphae with arbuscules common in the inner cortex. Fungal hyphae of *Glomus aggregatum* colonizing *Pisum sativum* (pea), penetrated cortical cells and differentiated into trunks and fine branches forming arbuscules upon reaching the inner cortex (Resendes et al. 2001).

Paris-type AM (Fig. 1.2.8.1 B) have extensive hyphae primarily growing intracellularly through cortical cells, with the fungus forming loops of hyphae (hyphal coils) inside each cell (Brundrett and Kendrick 1988). The hyphal coils are frequently found growing in the outer cortex, such as *Glomus coronatum* colonizing *Asphodelus* (Cavagnaro et al. 2001b).

A difference in hyphal growth rate of *Arum*- and *Paris*-type AM was found in many studies. *Lycopersicon esculentum* (tomato) colonized by *Glomus intraradices* displaying *Arum*-type AM reached 95% colonization within 16 days (Rosewarne et al. 1997). In the study of Cavagnaro et al. (2001) with similar pot culture conditions, *Allium porrum* L. and *Asphodelus fistulosw* L. both inoculated with *Glomus coronatum* displayed *Paris*-type AM only reaching about 50% colonization even after 25 days. Brundrett et al. (1985) proposed the idea that lower resistance of intercellular air spaces facilitated the faster growth of *Arum*-type hyphae compared with the

intracellular path in *Paris*-type, but this has not been tested stringently.

Smith and Smith (1997) first suggested the word "intermediate" morphotype, meaning a mixture of *Paris*- and *Arum*-type morphologies to indicate the AM complexity. Dickson (2004) conducted a survey of 12 plant species each colonized by six AMF species and showed that there was a continuum of mycorrhizal structures ranging from *Arum* to *Paris*, depending upon both the host plant and fungal species. They suggested that *Paris*- and *Arum*-types represented the extremes of a range of morphologies, whereas intermediate was a term to describe structures which were in between *Arum*- and *Paris*- morphologies.Yamato (2004) also found that 20 out of 26 Poaceae plants had both *Arum*- and intermediate-type AM.

In summary, these studies showed that the fungal morphologies were more complex than previously thought, and that it was difficult to use a simple classification scheme to distinguish *Arum*-, *Paris*-, or intermediate morphotypes. Also, the classification of fungal morphology may vary depending upon the interpretation by the investigator.

1.2.8.2. Factors involved in determining AM morphotypes

1.2.8.2.1. Plant genome has main control over AM morphotypes

Depending on the plant species colonised, the same AM fungal species is capable of producing either *Arum*- or *Paris*-type structures. In the study of Ahulu et al. (2006), *Hedera rhombea* (Miq) Bean, *Rubus parvifolius* L., and *Rosa multiflora* Thunb. were inoculated with five fungal species (*Acaulospora longula*, *Glomus claroideum*, *Glomus etunicatum*, *Gigaspora rosea*, *Scutellospora erythropa*) as single inocula. All fungal treatments produced *Paris*-type AM in *H. rhombea* but *Arum*-type AM in *R. parvifolius* and *R. multiflora*, indicating that AM morphology may be strongly controlled by the identity of the host plants.

Brundrett and colleagues (Brundrett and Kendrick 1988; 1990a; 1990b, Brundrett et al. 1985; 1990) published a survey that identified AM morphotypes of a large number of plant families. Later, Smith and Smith (1997) reviewed their work and found in summary 39 families with *Paris*-type, 26 with *Arum*-type, and 21 with either intermediate or both morphologies. Dickson et al. (2007) compiled all their data and found that, among the angiosperms, both *Arum*- and *Paris*-types and their combinations occur frequently with one predominant type in individual families. In the monocots, nine families have been recorded with only *Arum*-type, eight families with only *Paris*-type AM, and eight families with intermediate type. For example, Alliaceae is reported as predominantly *Arum*-type. In the dicots, the picture is similar. For example, in the asterids, one of the largest dicot groups, eight families have only *Arum*-type AM, about six families have only *Paris*-type AM, and the rest, both types. Among the 51 genera of Asteraceae investigated, 49 of them are recorded with *Arum*-type AM. These data support the conclusion that the plant genome, not the AM fungal genome, has the main influence on AM morphology.

Continuous longitudinal air spaces (AS) in the root cortex might be a factor influencing the formation of AM morphotypes under the assumptions that: 1) Long intercellular spaces of the cortex in some plants might provide a pathway of low physical resistance to fungal growth, leading to rapid sequential penetration of cortical cells and growth of arbuscules; 2) *Paris*-type AMF grow intracellularly in the absence of air-spaces; and 3) the occurrence of limited or discontinuous intercellular spaces in roots or differences between outer and inner cortex might account for intermediate structures (Brundrett and Kendrick 1988, 1990a; Smith and Smith 1997). However, Ahulu et al. (2007) found that *Voyria obconica* Prog. formed *Paris*-type AM in spite of the presence of intercellular spaces in the cortex, which indicated that root anatomy was not the only determinant. Previous studies on root anatomy focused on describing their anatomical structures, while little quantification work has been reported for the distribution of air-spaces (AS) in root cortex. My study will examine whether there is potential correlation between the quantity of AS and AM morphotypes.

1.2.8.2.2. Fungal identity of AM morphotypes

Fungal species were also found influencing AM morphology. AM showed varied structures in the same host, as in *Trifolium subterraneum* (Abbott 1982), Gentianaceae (Demuth et al. 1991) and *Lycopersicon esculentum* (tomato) (Cavagnaro et al. 2001a). Among the six different AM fungal species inoculating tomato, three (*Glomus intraradices, Glomus mosseae, Glomus versiforme*) formed *Arum*-type AM, whereas the remaining three species (*Gigaspora margarita, Glomus coronatum, Scutellospora calospora*) formed *Paris*-type (Cavagnaro et al. 2001a). Later, Smith et al. (2004) found that, in the same tomato cultivar, *Gigaspora rosea* formed *Paris*-type AM structure and *Glomus caledonium* formed intermediate AM structure. This result demonstrated that, in this tomato cultivar, fungal species were also important in determining AM morphologies. Kubota et al. (2005) also showed, with a different tomato cultivar, that both types of colonization could be produced in the same root system with mixed fungal inocula. They assumed this due to infections from different fungal species, as a root can be colonized by more than one AM fungus in the field.

Bonfante and Perotto (1995) suggested that penetration of host roots was due to a combination of mechanical and enzymatic mechanisms. Fungal cell wall components, such as melanin, were able to trap solutes to increase osmotic gradient and then induced increasing hydrostatic pressures (Howard and Ferrari 1989). This mechanism showing the ability to exert hydrostatic pressures may apply to the penetration of cortical cell walls to form the *Paris*-type AM. Low and regulated production of fungal cell wall degrading enzymes were able to cause extensive wall degradation. Investigations on *Glomus mosseae* have demonstrated the production of small amounts of cell wall degrading enzymes such as pectinase and cellulase (Garcia-Romero et al. 1991). Therefore, varied enzymatic and mechanical characteristics of different fungal species may be a factor affecting their morphotypes in host roots.

1.2.8.2.3. Other factors determining AM morphotypes

Environmental conditions such as soil properties, temperature and light were reported to affect the abundance of hyphal colonization (Smith and Read 1997). There is no evidence showing the correlation of the rates of root growth and (via differences in soil aeration) extent of intercellular spaces with AM morphotypes to date. Dickson et al. (2007) suggested that various factors influence AM morphotypes, where the plant genome was most prominent, followed by a possible role of the AM fungal genome, as well as plant/AMF interactions via host/fungus preferences and environmental conditions.

1.2.9. Impact of AMF in ecosystems

AMF in soils plays an important role in the maintainance of the biodiversity of an ecosystem (van der Heijden et al. 1998). AM can increase drought tolerance of wheat in response to water stress (Goicoechea et al. 1998) and exert protective effects on plants growing on polluted soils. Christie et al. (2004) found that AM alleviated metal toxicity, particularly Zn toxicity, and they suggested that binding of metal in mycorrhizal structures and immobilization of metals in the mycorrhizosphere may contribute to the protective effects. Rivera-Becerril et al. (2002) found that *Glomus intraradices* attenuated the toxic effect of cadmium in pea. Mycorrhizal plants are also employed as a revegetation tool to modulate desertification in arid regions. Mathur and Vyas (1999) found that *Glomus constrictum*, a common fungus of arid and semi-arid regions, significantly improved the nutrient uptake and biomass production of the host plant.

Soil quality is influenced by AMF through their forming water-stable soil aggregates (Andrade et al. 1998; Bethlenfalvay et al. 1999; Miller 2000). AMF produces a very stable hydrophobic glycoprotein, glomalin, which is deposited on the

outer hyphal walls of the extraradical mycelium and on adjacent soil particles. Glomalin appears to act as a long-term soil binding agent (Wright and Upadhyaya 1998, 1999) by coating fine soil particles with a layer of hydrophobic materials and by gluing the particles together. Glomalin creates microanaerobic sites to protect the organic matter from oxidation and microbial attack (Robson et al. 1994). AMF is regarded as an essential component of an ecosystem and is important in maintaining soil structure.

1.3. The AM fungi (AMF)

1.3.1. AMF classification

Classical techniques for AMF identification are based on the morphology of the spore, especially spore wall structure and spore development (Bentivenga and Morton 1994; Franke and Morton 1994). Although approximately 200 AM forming species are described to date (online electronic taxonomic guides/keys invam.caf.wvu.edu), the majority of species remain undescribed (Bever et al. 2001). A major difficulty in field samples is that low spore number, parasitization of spores, age, environmental alteration of spores (e.g., discolouration) and season-dependent spore density (Schultz et al. 1999) will hinder or even mislead accurate identification (Bever et al. 2001).

An alternative approach that avoids the limitations of morphological criteria is the direct analysis of fungal nucleotide sequences. Ribosomal RNA genes are ideal for this application because regions of conservation and variability facilitate sequence alignment and comparison, respectively. For example, the characterization of isolates in *Gigaspora* from different geographical areas was accomplished by both morphological and molecular methods (Lanfranco et al. 2001). The complementary analysis can clarify relationships among species of low morphological divergence.

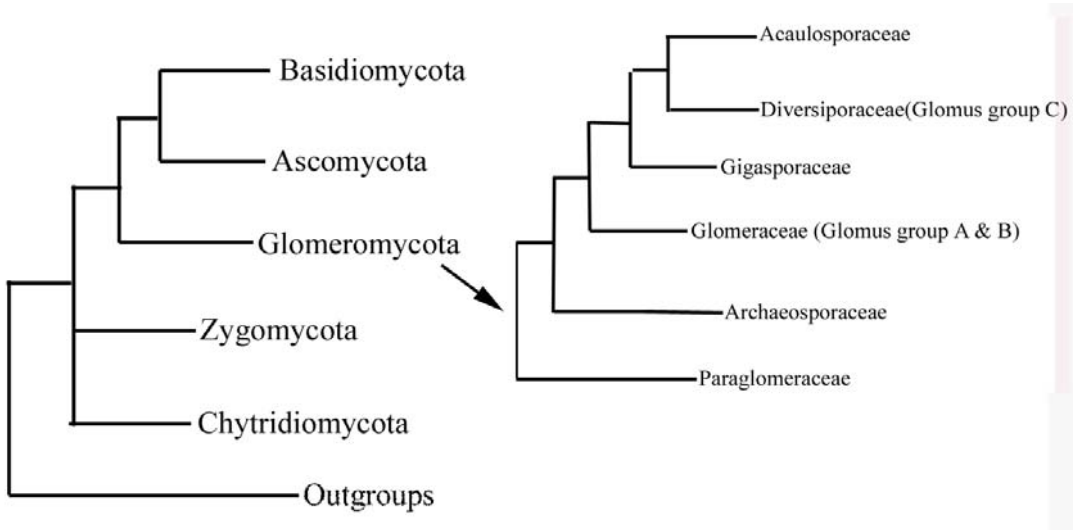
Although the classic approach is time consuming and subjective, the systematics is

well recorded and understood. With the advent of molecular techniques, the classification of AMF has undergone major revisions, even though there are still some limitations (discussed in 1.3.5.4.).

1.3.2. Current state of AMF taxonomy

With the advent of molecular taxonomy methods, the taxonomy of the kingdom Fungi is in a state of revision. Under earlier classification, AMF were placed in the class Glomeromycetes within the division Zygomycota. Today, these relationships are being reassessed by nucleic acid sequence analysis, with special emphasis on the nuclear DNA encoding the 18S rRNA gene. Based on genetic evidence, AMF are placed into their own phylum, the Glomeromycota.

Fig.1.3.2. Current phylogenetic tree of fungus (left) based on sequence analysis of 1419 bp of small subunit rDNA sequences and the phylum Glomeromycota (right) based on 1539 bp of small subunit rDNA sequences. *Neurospora crassa* was used as outgroup. Adapted from Redecker and Raab (2006).



Above the genus level, the family Glomeraceae was erected by Pirozynski and Dalpe (1989). Gigasporaceae and Acaulosporaceae were established by Morton and Benny (1990). Morton and Redecker (2001) described two new families, Archaeosporaceae and Paraglomaceae, to comprise deeply divergent lineages of AM fungi. Paraglomeraceae is so ancient that it does not cluster with the rest of the Glomeromycota. Schussler et al. (2001) divided the new phylum Glomeromycota into the orders Glomerales (families Glomeraceae *Glomus*-groupA, Glomeracea *Glomus*-group B), Diversisporales (families Gigasporaceae and Acaulosporaceae), Archaeosporales (families Archaeosporaceae and Geosiphonaceae) and Paraglomerales (family Paraglomaceae). Together with the corresponding new genus *Diversispora*, the family Diversisporaceae was erected by Walker and Schussler (2004).

1.3.3. Genetic diversity of spores and hyphae

AMF have an aseptate, multinucleate mycelium, which originates from asexual, multinucleate spores. The DNA content of AMF spores ranges from 0.13 to more than 1.00 pg per nucleus, which is larger than that of other fungi (Bianciotto and Bonfante 1992; Hosny et al. 1997). The number of nuclei found in individual spores varies according to species but ranges between 1000 and 20,000 (Becard and Pfeffer 1993; Viera and Glenn 1990) with 1000-5000 accepted as most likely. There are about 90 copies of ribosomal RNA (rRNA) genes for each nucleus (Passerieux E 1994). The enormous numbers of nuclei in spores are the source of genetic variation between different AMF species and even within the same species (Bago et al. 1998; Clapp et al. 1999; Lanfranco et al. 1999; Sanders et al. 1995). Zezé et al. (1997) reported that *Gigaspora margarita* displayed genetic variation between spores by testing loci of satellite regions, which consist of short, repeating sequences of nucleotide pairs near the centromere. This intra-species variability of nuclear ribosomal genes complicates distinguishing closely related species, but it was not an issue at the generic or family

level (Redecker and Raab 2006).

However, experimental contamination may blur the understanding of fungal genetic diversity. The study of Hijri et al. (1999) showed strongly diverging sequences within single spores, but later Redecker et al. (1999) found it due to contamination by Ascomycota. Roots also contain endophytic fungi, mostly Ascomycetes (Redman et al. 2002; Rodriguez et al. 2004; Kauhanen et al. 2006). Schussler et al. (2003) pointed out that the process of spore extraction from the substrate could cause severe damage to spores and hyphae of AMF and other fungi. Consequently, the individual spores might be bathed in a mixture containing DNA from several species. Therefore, even single-spore DNA isolation protocols might be the cause of divergent sequences within in a single spore in the studies of Rodriguez et al. (2001) and Schwarzott et al. (2001).

1.3.4. *In vitro* culture of AMF

AMF are obligate biotrophs; that is, they cannot be cultured in the absence of an intimate relationship with host plant roots. The obligate nature of the AMF hampers the study of these ubiquitously and fundamentally important fungi. Mosse and Hepper (1975) first reported *in vitro* co-culture between a root organ culture and an inoculum from a glomalean species. Later, Becard and Fortin (1988) modified the experimental conditions for AM monoxenic cultures, using Ri T-DNA transformed carrot roots (Mugnier and Mosse 1987) as the host. The monoxenic culture technique provides a reliable way for growth, sporulation and long-term maintenance of AMF. It comprises three steps (Becard and Fortin 1988): 1) the choice of an adequate starter inoculum and its isolation; 2) the disinfection of propagules; 3) association with a susceptible host root. St-Arnaud et al. (1996) modified this technique by using bi-compartmented petri plates to separate AM fungal extraradical mycelium from the host root and its environment.

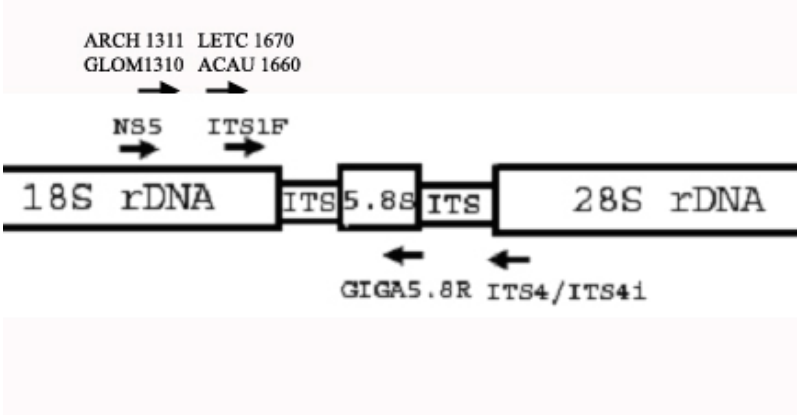
From then on, the AM monoxenic culture technique is combined with cytology, biochemistry, physiology (Bago et al. 1999, 2000, 2002, 2003, 2004; Jolicoeur et al. 1998; Pfeffer et al. 1999) and molecular taxonomy (Lammers et al. 2001; Simon et al. 1992, Simon et al. 1993) for further study of AMF. However, monoxenic culture cannot maintain all fungal species and might cause potential loss of strain characteristics during successive transfers, thus not showing all fungal phenotypes.

1.3.5. Molecular identification of AM diversity

1.3.5.1. Target DNA sequences

The majority of nucleic acid information derived from the Glomales is from ribosomal DNA (rDNA), which code for ribosomal RNA (rRNA) (Redecker 2000). rDNA genes are present in multiple copies arranged in tandem arrays, with each repeat unit consisting of a small subunit (SSU) or 18S rDNA and a large subunit (LSU) or 28S rDNA gene separated by an internal transcribed spacer (ITS), which are located between the 18S and 5.8S rDNA coding regions (ITS1) and between the 5.8S and 28S rDNA coding regions (ITS2) (Roderick and Edward 1998). The coding regions of 18S, 5.8S and 28S rDNA genes evolved slowly, and are relatively conserved among fungi. The non-coding ITS1 and ITS2 regions evolved more rapidly, leading to sequence variability among genera and species of fungi (Chen et al. 2000).

Fig.1.3.5.1.The amplification sites of universal primers and group-specific primers.



1.3.5.2. Primer design

Simon et al. (1992) designed the first specific PCR primer for AMF: VANS1, based on the data obtained from the 18S SSU sequences of ribosomal DNA using PCR fragments generated with universal eukaryotic primers. Later, Simon et al. (1993) designed other group-specific primers (VAGLO, VAACAU, VALETC and VAGIGG) to amplify unknown taxa from plant roots. As more SSU sequences became available, it was found that the VANS1 priming site was not well conserved in all groups of the AMF (Clapp et al. 1999; Redecker 2000; Schussler et al. 2001). The ITS region is extensively used for molecular taxonomy. ITS1 and ITS4, published by White et al. (1990), were used to obtain sequence information from spores collected from the field (Sanders et al. 1995) and to elucidate the relationship among AMF species (Redecker et al. 1997). Later, Redecker (2000) designed group-specific primers for five major phylogenetic lineages of AMF to amplify the highly variable ITS. The group-specific primers worked well for AMF collected in field (Redecker et al. 2003; Wubet et al. 2003; Hijri et al. 2006; Shepherd et al. 2007). In my study, Redecker's group specifc primers will be used to detect fungi from family level in specimens collected from field soil.

1.3.5.3. Polymerase chain reaction (PCR)

PCR technique is prominent for its efficiency to amplify small quantities of the targeted nucleic acid sequence from extracts (Schwarzott and Schussler 2001; Van Tuinen et al. 1998). However, PCR inhibitory substances in soils will interfere with amplification and subsequent analysis (Van Tuinen et al. 1998), leading to misleading amplification. Sample purification by polyvinyl polypropylene (PVPP) (Berthelet et al. 1996) or dilution of samples (Schwarzott and Schussler 2001) can be employed to reduce the inhibitory effect. Also, the template tends to form its own favorable secondary structure (Gelfand and White 1990), which will lead to a preferentially annealing template during PCR amplification. Thus, a bias of the PCR product will

mislead the interpretation of AMF species identified in samples. The use of bovine serum albumin (BSA) helps to prevent excessive secondary structure formation during the annealing phase of PCR. In summary, PCR technique is widely used in assessing AMF diversity, but it is not without potential artifacts.

1.3.5.4. Limitations

Molecular methods allow the identification of AMF independently of morphological criteria, especially when the colonization is low. The PCR technique successfully increases small quantities of DNA to sufficient quantities for analysis. However, the lack of understanding of genetic polymorphism in AMF reduces the confidence in the molecular analysis. One single spore can contain considerable variation in ITS and 5.8S rDNA sequences (Redecker et al. 1999). Genetic variation is found not only between different species, but as well within the same species (see section 1.3.3 for a discussion of some fators). Therefore, the single-sequence, single-species hypothesis may not fit with AMF (Dodd et al. 2000; Schussler et al. 2001). Redecker et al. (2003) suggested that molecular analysis of AMF primarily identifies sequence groups, not species. Furthermore, AM significance in ecosystems must be related to its abundance. But molecular methods cannot reflect the fungal abundance, compared with morphological analysis. Nevertheless, molecular analysis, in combination with the classic morphological characterization of spores, would contribute to a better understanding of AM communities within roots.

1.4. Fine endophytes (FE)

Fine endophytes (FE), formerly classified as *Rhizophagus tenuis* (Greenall 1963), were reclassified as *Glomus tenuis*, supported by taxonomic analysis (Hall 1977). The hyphal morphology of FE differs somewhat from other *Glomus* species and even other genera in Glomales. The hyphal diameter of FE is less than 1μm (Hooker et al. 2007; Smith and Read 1997), and the spores are too small (10 μm) to separate from

soil by standard methods (Brundrett et al. 1996). The structures of hyphal coils, swellings and projections have been reported (Nicolson and Schenck 1979; Thippayarugs et al. 1999; Allen et al. 2006), but the shape, size and position of vesicles were variable in different studies. Wang et al. (1985) found that colonization of FE was linked with soil pH. In acidic soils, roots were found to be highly colonized by FE, and the proportion of fine and coarse endophytes decreased with increasing soil pH. FE were found beneficial for its host plants in especially low nutrient conditions (Crush 1973; Rabatin 1979), which was consistent with their apparent abundance in generally nutrient-poor arctic soils, as reported by Ormsby et al. (2007) for Asteraceae in Arctic Canada. But the mechanism of FE nutrient transfer is not well known, but Gianniazi had shown that at least nutrient transfer was related to FE arbuscules (Gianinazzi-Pearson et al. 1981).

1.5. Septate endophytes (SE)

In addition to mycorrhizal fungi, roots are also commonly associated with other endophytic fungi. Septate endophytes (SE), which can include conidial or sterile ascomycetous fungi, are found colonizing roots of a wide range of plants from the tropics to Arctic and Alpine habitats (Jumpponen and Trappe 1998). Plant species in cool, nutrient poor, arid, alpine or sand dune ecosystems are reported to have higher incidence of SE (Barrow and Aaltonen 2001; Hambleton and Currah 1997; Haselwandter and Read 1982; Read 1991; Stoyke et al. 1992). It is common for plant species to have both typical mycorrhizal and SE fungal associations (Peterson et al. 2004). Furthermore, SE could be of equal or even higher incidence than AMF, as reported by Barrow et al. (1997) and Ormsby et al. (2007). Prevalence of SE rather than AMF was also found in *Carex* sp. in subarctic alpine regions (Haselwandter and Read 1982; Ruotsalainen et al. 2002). This widespread nature of SE in various ecosystems and their mutualistic response to host plants, such as increased growth and phosphorus uptake, suggest that SE are of significant importance in ecosystems (Barrow and Osuna 2002; Haselwandter and Read 1982; Jumpponen 2001). As well

as for mutualistic characteristics similar to mycorrhizas, SE may function as pathogens or saprophytes as well (Jumpponen 2001). *Potentilla fructicosa* shoot biomass decreased when inoculated with two strains of SE *Leptodontidium orchidicola* (Fernando and Currah 1996). Studies by Stoyke and Currah (1993) and Wilcox and Wang (1987) showed a clearly pathogenic association between *Phialocephala fortinii* and the host plant. Therefore, the influence of SE to host plants is not of simply mycorrhizal benefit, but more complex under different plant-fungus combinations and environmental connections. Melanization of SE and microsclerotia may give them a characteristic dark colour that is easily visible under light microscopy. Melanized hyphae are observed most frequently growing both inter- and intracellularly within the cortex and the epidermis as well as external extensions on the root surface. Further experimental studies are needed to elucidate the classification of SE and to draw any conclusions concerning their beneficial relationship with host plants.

1.6. Microscopy application in the morphology and quantification study

Most studies of AM morphology routinely stained roots with biological stains such as trypan blue, chlorazole black E (CBE) and lactofuchsin (LF), which could bind to chitin, a component of most fungal walls (Bevege 1968; Kormanik et al. 1980), as well as components of plant cell walls, lignin and cellulose. Transmitted light microscopy was used to observe the mycorrhizal structures in host roots. However, these techniques may lack sufficient contrast to resolve details particularlly for FE (Allen et al. 2006). Therefore, different microscopies are employed together to study the mycorrhizal structures.

1.6.1. Confocal laser scanning microscopy (CLSM)

CLSM has several advantages over conventional optical microscopy when examining fluorescent specimens. CLSM is capable of collecting serial optical

sections from thick specimens and it uses spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus. Thus, it provides sharper images of 2D and 3D objects than can be obtained with conventional optical microscopes. Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime. For the visualization technique described in Allen et al. (2006) and Ormsby et al. (2007), root samples need to be cleared of cytoplasm and then stained in acid fuchsin in lactic acid, followed by a destaining step. Stained roots were mounted on slides and imaged at specific excitation related to the fluorescent stain. The LF-stained chitin in AMF fluoresces when exposed to green light. For CLSM, the Zeiss software is capable of reconstructing 3D images by linking series of images taken at different focal depths. Thus, it can be easier to locate the fungal structures within the host root.

1.6.2. Transmission electron microscopy (TEM)

The principles governing image formation in a transmission electron microscope (TEM) are similar to those of the light microscope, the major difference being that the specimen is imaged with an electron beam whose wavelength is about a thousand times shorter than that of visible light; this results in a greater resolving power. The electron beam is focused by electromagnetic lenses through an ultrathin specimen in a vacuum. The image is enlarged progressively in a further series of lenses and finally projected onto an electron-sensitive photographic emulsion. TEM is widely employed in AM morphology studies (Gross et al. 2003; Kinden and Brown 1975). The standard procedure of TEM consists of sample fixation, embedding, sectioning and staining (details discussed in Methods and Materials). The resolution of TEM is high enough (< 1.0 nm) to provide ultrastructural information about AMF colonizing within cortical cells of root, revealing the membranous structures of fungus-root interface. However, detail is inversely proportional to sample size.

1.7. Study objectives

AM morphology and abundance are two key indices of description of AM in host roots. AM morphologies are more complex than the orignally defined *Arum*- and *Paris*-type AM. Dickson et al. (2007) emphasized recording the structural classes of AMF during the survey of AM diversity. In my study, wide field and laser confocal microscopy have provided more details about the diverse AM morphologies. Ormsby et al. (2007) modified the method of McGonigle et al. (1990) to quantify AM abundance as well as other types of endophytic relationhship. The abundance data will be used in the analysis of my study objectives. The soil properties (pH, organic carbon, extractable phosphorus) are also assessed to examine their potential effects on AM abundance.

1.7.1. Relationship of root anatomy and AM morphotypes

Many studies have shown that AM morphotype is influenced by both host plants and AMF, but information about the relationship between the root anatomy and AM morphotype has been lacking. One of my objectives was to evaluate the contribution of host root cell packing to AM morphotypes. *Arum*-type AM have extensive intercellular hyphae with highly branched intracellular arbuscules preferentially growing in the inner cortex, whereas *Paris*-type AM have extensive hyphae growing intracellularly through cortical cells, with hyphal coils primarily in outer cortex. A mixture of mycorrhizal structures ranging from *Arum*- to *Paris*-types was named as intermediate morphotype. *Paris*-type AM are found growing intracellularly in the absence of air-spaces. Intercellular air spaces of the cortex in some plants might provide a pathway of low physical resistance to fungal growth, which could facilitate the growth of *Arum*-type AM. The occurrence of limited or discontinuous intercellular air spaces in roots or differences between outer and inner cortex might account for intermediate structures.

I examined the fungal colonization status of two plant species: dandelion (*Taraxacum officinale,* Asteraceae) and chive (*Allium schoenoprasum,* Alliaceae) and preliminary evidence showed they had both AM morphotypes. Transverse sections of root samples were used to provide information about the location of hyphae (inter- or intracellular), which provide better pictures of the fungal distribution of *Arum*-, *Paris*- and intermediate morphotypes within host root. Also transverse sections are used to calculate the proportion of air spaces of inner/outer cortex, by which to investigate the possible relationship of root anatomy and AM morphotypes.

1.7.2. Diversity of AM communities in root system

My second objective was to use molecular techniques to study the diversity of AM communities in the roots of dandelion (*Taraxarum officinale*) and chive (*Allium schoenoprasum*). My CLSM studies and evidence from the literature (Cavagnaro et al. 2001a, Kubota et al. 2005) showed that AM morphologies were not simple as defined *Arum*- and *Paris*-types. Intermediate AM morphotype, as proposed by Smith and Smith (1997) was also prevelant in the roots of dandelions and chives of my study. In order to better understand if the AM morphotypes are caused by different fungi or by the same fungus growing in different ways, it is necessary to identify the fungi within the roots.

II. MATERIALS AND METHODS

2.1. Soil analysis

2.1.1. pH

Soil pH is measured using 0.01M $CaCl_2$ solution (Thomas 1996). This gives more consistent results than using water. When the soil is suspended in water, most of the H^+ ions tend to remain attracted to the soil particles and are not released into the solution. The addition of small amounts of $CaCl_2$ provides Ca^{2+} ions to replace some of the H^+ ions on the soil particles, forcing hydrogen ions into solution and making their concentration closer to that found in the field where there are always Ca^{2+} and other ions dissolved in the soil solution. Therefore, the pH measured in $CaCl_2$ solution is always lower than the pH measured in water. The pH of fresh soil was measured in the following suspensions: 1:1 mixtures of fresh soil weight to volume of 0.01M $CaCl_2$ in double distilled water. All pH readings were taken using calibrated pH meter (VWR 8100 model) after shaking soil solution for 30 min. Three replicates of pH measurement were done to garden and roadside soil samples.

2.1.2. Organic matter

Total carbon was presumed to equal organic carbon in the soil samples, which were collected from non-calcareous sites in this study. The amount of organic matter is determined by a dry combustion method (Leco CR-12 carbon determinator, LECO Corporation, St. Joseph, MI, USA) whereby soil is heated in a stream of purified oxygen to > 900^oC and organic matter is converted to CO_2 and water. This combustion results in a change in weight and then the CR-12 measures the value of the organic matter in samples.

2.1.3. Extractable phosphorus

The test of available phosphorus was done by the ALS Laboratory Group in Saskatoon, SK (www.alsglobal.com) using the method of Olsen et al. (1954). A 1.0 gram sample of soil and 20 milliliters of 0.5 molar sodium bicarbonate ($NaHCO_3$) solution are shaken for 30 min. Blue color in the filtered extract is developed with successive additions of an ammonium molybdate-sulfuric acid solution and then an ascorbic acid solution and measured with a fiberoptic probe colorimeter at 882 nm. Results are reported as parts per million (ppm) in the soil. Treatment replicates for each sample were first subjected to analysis of mean, followed by mean comparisons by one-way ANOVA, then Fisher PLSD analysis to calculate the significance of differences.

2.2. Plant sampling

Dandelion (*Taraxacum officinale*) and chive (*Allium schoenoprasum*) samples were harvested with soils from two different soil types and times (Table 1.). The harvested plants and soils were kept in pots in greenhouse. In order to sustain the natural soil pH, the plants were watered with rainwater. Dandelions have a taproot root system and chives have as fibrous root system. Only roots confirmed to be attached to the dandelion taproot were used for analysis. Chive roots shown to be atteched were excised from fibrous roots. Roots were fixed in 3.7 % formaldehyde containing 0.5 % ethanol, buffered to pH 7.0 in 50 mM sodium-potassium phosphate.

Table 2.2. Sample location, date and soil type

Plant	Date	Location	Soil color, texture, compaction	Cultivated
Dandelion 1[a]	06-10	Garden	dark brown, loam, loose	+
Dandelion 2[a]	06-10	Garden	dark brown, loam, loose	+
Dandelion 3[a]	06-10	Roadside	light brown, sandy, dense	–
Dandelion 4[a]	06-10	Roadside	light brown, sandy, dense	–
Chive 1	06-05	Garden	dark brown, loam, loose	+
Chive 2	06-08	Garden	dark brown, loam, loose	+
Chive 3[a]	06-10	Garden	dark brown, loam, loose	+
Chive 4	06-10	Garden	dark brown, loam, loose	+

a: samples used for molecular identification

2.3. Detection of AMF by bright field microscope and CLSM

The fixed roots were subsampled following the method described by McGonigle et al. (1990). The roots were cut into 1-cm-long segments and dispersed in a large volume of water. The water was stirred to suspend fragments, and for each plant sample, 15 to 20 root fragments were collected as subsamples.

Selected root segments were cleared of cytoplasm by autoclaving in 10 % KOH for 20 min. KOH was removed with two washes in room-temperature 70 % ethanol. Cleared roots were stained for 3 h at 68 ºC in 0.05 % acid fuchsin dissolved in 85 % lactic acid (LF) (Ormsby et al. 2007) or 0.05 % chlorazole black E (CBE) in 1:1:1 distilled water: 85% lactic acid: glycerol (DLAG) (Allen et al. 2006). Roots were destained in DLAG at 47 ºC first for 20 min and then changed to fresh DLAG overnight. Stained roots were mounted in polyvinyl alcohol glycerol (PVAG) medium which was then polymerized overnight at 40ºC. Finally the slide edges were sealed with nail polish (Ormsby et al. 2007).

Endorhizal fungi were imaged using a Zeiss META 510 CLSM (www.zeiss.com) equipped with 25 x Plan NeoFluar N. A. 0.8 and 63 x C-Apochromat N. A. 1.2 multi-immersion objectives, each with differential interference contrast optics. Imaging used 543 nm excitation, 9.9 % intensity of a 25 mW beam from a HeNe laser, a HFT 488/543 beam splitter, and a 604-657 nm emission filter. Fluorescence and transmitted light images were collected simultaneously. Some images, and endorhizal quantitation, used a Zeiss Axioplan microscope equipped with a 20 x N. A. 0.5 Plan Neofluar, a 40 x N. A. 0.75 Plan Neofluar, and a 63 x N. A. 1.4 Plan Apochromat oil immersion objective, each with differential interference contrast optics. Axioplan wide-field epifluorescence imaging of lactofuchsin-stained material used a BP546 excitation filter, FT580 dichroic mirror, and LP590 emission filter. Images were captured using a Sensys CCD (www.roper.com) driven by ImageManager RSI.

2.4. Analysis of fungal abundance

Fungal colonization of root systems was assessed using the Multiple Quantitation Method (MQM) (Ormsby et al. 2007) and LF stained roots. Roots were examined using wide-field epifluorescence microscopy. Intersections were taken 1-2 mm apart and examined using 400 x total magnifications. Between 150– 200 intersections were taken for each sample. Each intersection was assessed individually for several types of endophyte: 4-6 μm wide aseptate hyphae characteristic of AM; arbuscules and vesicles associated with AM hyphae; 1- 1.5 μm wide aseptate hyphae characteristic of FE; SE hyphae; intercellular hyphae and hyphal coils. Intersections not associated with any fungi were scored separately to provide an estimate of colonization frequency corresponding to the ratio of colonized versus non-colonized root fragments. Results were reported for the root systems of individual plants and were expressed as mean ± standard error of the mean. Statistical analysis used Statview 1.01 to compare colonization abundance by one-way ANOVA followed by Fisher PLSD.

2.5. Transmission Electron Microscopy (TEM)

2.5.1. Fixation, embedding and sectioning

The diameter of sampled roots was less than 1 mm, which makes routine free-hand sectioning difficult. Thus, the roots were fixed for microtome sectioning instead. Freshly harvested roots were cut into 1-mm segments and fixed for 2 h in 3 % glutaraldehyde in Na/K phosphate buffer (0.1 M, pH 7.2), and then postfixed for 1 h in 1.0 % osmium tetroxide in the same buffer. After rinsing in distilled water, postfixed roots were dehydrated in a graded ethanol series using 20 % increments, 20 min each step, ending in two changes of 100% ethanol. Then the roots were gradually transferred to pure ice-cold propylene oxide, using three changes 20 min each time.

Glutaraldehyde-osmium tetroxide fixed root fragments were embedded in a 1:1

mixture of propylene oxide and freshly made liquid Epon resin (2 g Epon812: 1 g DDSA: 1 g NMA: 0.07 mL DMP-30) after which the propylene oxide was allowed to evaporate overnight. Samples were transferred to freshly made Epon resin in plastic embedding molds and baked in a 60°C oven for 2 d. Specimens were trimmed and sectioned on a Reichert-Jung microtome. Both semi-thin (900 nm) and ultra-thin (70 nm) sections in cross and longitudinal orientation to the root axis were cut with a glass knife. Glass knives were made with LKB-Bromma 7800 knifemaker. All chemicals were purchased from VWR.

2.5.2. Section staining and observation

The 900 nm semi-thin cross sections were stained with toluidine blue (0.5 g toluidine blue: 100 mL water: 1 g sodium borate). Images of semi-thin sections were captured by a Zeiss microscope equipped with 25 x Plan NeoFluar N. A. 0.8 objective.

The 70 nm ultra-thin sections were transfered to 200-mesh copper grids and stained with 2 % aqueous uranyl acetate (0.4 g uranyl acetate: 10 ml methanol: dH_2O 10ml) for 20 min, followed by 0.4 % lead citrate (0.1 g lead nitrate: 0.1 g lead acetate: 0.1 g lead citrate: 0.2 g sodium citrate: 8.2 ml dH_2O: 1.8 ml 4% NaOH, pH 12) for 10 min (Sato 1968). Sections were examined on a Philips CM10 TEM used at an accelerating voltage of 60 KV. Images of ultra-thin sections were collected on 3.25 x 4 inch Kodak electron microscope film. Exposed films were developed in Kodak D-19 Developer solution for 2 min then rinsed with water. The developed films were fixed in Kodak Film Fix solution for another 5 min again followed by water washes. Once dried, the negatives were digitized and stored electronically as Adobe Photoshop 7.0.1 documents.

2.6. Root anatomy analysis

Images of semi-thin sections were analyzed using Image J 1.33U software. The cortex region was divided into outer (two or three cortical cell layers adjacent to the epidermis) and inner (two or three cortical cell layers adjacent to the stele) cortex. The air space (AS) percentages of the inner, outer and whole cortex region were calculated separately by adding up the intercellular areas within one region and then dividing by the total area of that region. The aim to distinguish the inner and outer cortex is to investigate whether there is any relationship between fungal structure and the quantity of AS in different regions of cortex. Results were reported for the root systems of chive and dandelion and were expressed as mean ± standard error of the mean. Statistical analysis used Statview 1.01 to compare air-space percentages by one-way ANOVA followed by Fisher PLSD.

2.7. DNA extraction

2.7.1. Bead beating method

Fresh root samples (0.5 g) were mixed with 2 µL proteinase K (20 mg/mL) (www.invitrogen.com) and 10 µL sterile sodium phosphate buffer (100 mM, pH 8.0). Roots were ground to fine mash with sterile mortar and pestle prechilled to –20 ^{o}C and transferred to a 1.5 mL microfuge tube. Then, 500 µL of sterilized sodium phosphate buffer (100 mM, pH 8.0) was added to each sample and mixed by inversion. 0.5 g sterile 0.5 mm zirconia/silica beads and 0.5 g sterile 1.0 mm zirconia/silica beads (http://www.biospec.com/Beads.htm) were added to the mixture. Sodium phosphate buffer was added if the root tissue could absorb more. The mixture was homogenized with a vortex at maximum speed for 1 min, incubated on ice for 1 min, followed by another 1 min vortex. Five hundred microlitres of 250 mM Tris-HCl pH 8.0 and 10 µL of proteinase K (20 mg/mL) were added, mixed with a flick, followed with another 100 µl 20 % SDS and mixed by inversion. The 1.5 mL microfuge tube was incubated for 1 hour at 37 °C water bath with inversion every 10 min. After 15

min, samples were centrifuged at 5600 rpm (Beckham, Germany) at room temperature. Four hundred microlitres clear supernatant was transferred to sterile 1.5 mL tubes. 200 µL 7.5 M ammonium acetate was added and mixed by gentle inversion. The mixtures were then incubated on ice for 15 min. Finally, 600 µL –20 ^{o}C 2-propanol was added to precipitate the DNA at –20 °C overnight. The extract was centrifuged 30 min at 4 °C at 7600 rpm. The supernatant was decanted and the pellet was washed with 1 mL cold 70 % ethanol followed by 10 min centrifuge at 4 °C. After air-drying the pellet, 20 µL TE pH 8.0 was added to dissolve the DNA.

2.7.2. DNA purification

The DNA extract was purified using acid washed polyvinylpolypyrrolidone (PVPP) column (Berthelet et al. 1996) to remove the protein, RNA and other impurities. PVPP slurried in 20mM potassium phosphate (pH 7.0) was loaded to the sterile microspin columns and spun for 3 min at 2900 rpm at room temperature. If the packed PVPP in the column was less than ¾ the height of the column, more of the slurry was added and spun for an additional 3 minutes, removing the liquid after spinning. The DNA extract was then loaded onto the center of the column being careful not to touch the side. This ensured that all of the sample would pass through the column and be cleaned, not run down the side of the column. The columns were then spun again for 3 min. The clean DNA extract was then stored at -20^{o}C and was used as template for PCR.

2.8. Polymerase chain reaction (PCR)

2.8.1. Nested PCR

“Nested” means that two pairs of PCR primers are used sequentially for a single locus. The second pair of primers is designed to bind within the first PCR product, so that the second PCR product shorter than the first one. Therefore, if the wrong locus is amplified by mistake, the probability is very low that the wrong product will also be

amplified by a second pair of primers. This technique is advantageous for amplifying specific DNA sequences from a complex mixture of DNA. In AMF studies, nested PCR starts with the first universal fungal primer pair to generate enough general fungal DNA and then second (nested) primers to target particular AMF templates. This technique is useful if the target DNA concentration is relatively low within the general population (Van Tuinen et al. 1998).

PCR was performed in a nested procedure as described by Redecker (2000), containing 10X PCR buffer, 2 mM $MgCl_2$, 50 μM (each) of dATP, dCTP, dGTP, dTTP, 0.2 μM each of the primers, 0.1 μl *Taq* polymerase (www.invitrogen.com) and genomic DNA. The first round of amplification was performed using the universal primers NS5, ITS4 (White et al. 1990) (Table 2.8.1) to amplify the rDNA region (Fig.1.3.5.1). An initial 3 min denaturation at 95 °C was followed by five cycles of 30 s at 95°C, 30 s at 52 °C, and 1.5 min at 72 °C. Thereafter, 25 to 30 cycles with annealing at 51 °C were performed. PCR products were run on agarose gels (0.7 %) to test if it accorded with the expected size of about 1200 bp. Amplified products were used as templates for second round PCR. The second round PCR was conducted seperately with five pairs of Glomales-specific primers (Redecker et al. 2003), which specifically targeted at different groups of AMF species (Table 2.8.1). Primer pairs were ARCH1311, ACAU1661, LETC1670, GLOM1310, each paired with ITS4i, and GIGA5.8R paired with ITS1F. The expect PCR product size was based on the accession from which they were designed (Table 2.8.2, Redecker 2000). The difference of the PCR condition compared with the first round is the annealing temperatures at 61 °C for 5 cycles then 60 °C for 25 cycles. PCR products were electrophoresed on 2 % agarose gels. DNA bands were estimated with the aid of a standard DNA ladder (Qiagen, ON, Canada). By comparing the bands of PCR product to the expected size, it provided preliminary classification of fungal groups tested.

Table 2.8.1. Primers used in nested PCR and their Tm.

Primers	Sequence (5'-3')	Tm (^{o}C) [a]
NS5	AACTTAAAGGAATTGAGGGAAG	57
ITS4	TCCTCCGCTTATTGATATGC	58
ITS4i	TTGATATGCTTAAGTTCAGCG	56
ITS1F	CTTGGTCATTTAGAGGAAGTAA	53
ACAU1661	TGAGACTCTCGGATCGGG	60
LETC1670	GATCGGCGATCGGTGAGT	62
GLOM1310	AGCTAGGCTTAACATTGTTA	50
GIGA5.8R	ACTGACCCTCAAGCAKGTG	58

a All Tms were supplied by Invitrogen, Canada.

Table 2.8.2. Five primer pairs (Redecker 2000) and the size of expected PCR products in nested PCR.

Primers pair	Product size (bp) [a]	Target group[b]
ARCH1311-- ITS4i	1052	*Archaeospora gerdemannii / trappei group*
		Glomus occultum / *brasilianum* group
ACAU1661--ITS4i	645	Acaulosporaceae
LETC1670-- ITS4i	676	*Glomus etunicatum / claroideum* group
GLOM1310-- ITS4i	1012	*Glomus mosseae / intraradices* group
GIGA5.8R-- ITS1F	305	Gigasporaceae

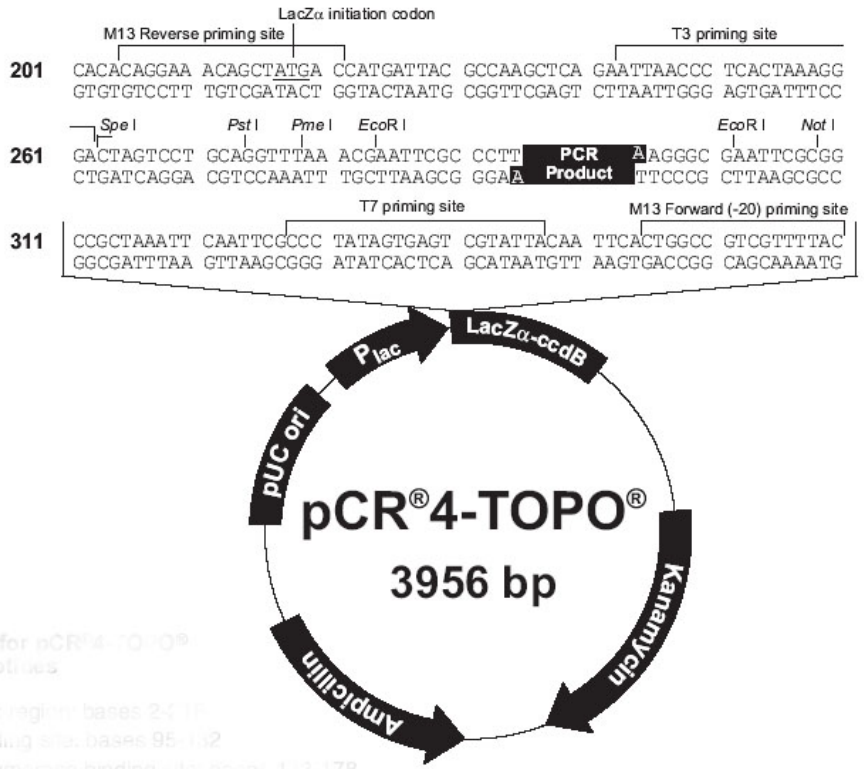
a: Expected PCR product size are based on the accession from which they were designed. b: Groups as defined in Redecker (2000).

2.9. Cloning and sequencing

2.9.1. TOPO TA cloning advantages

Taq polymerase has a nontemplate-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized pCR®4-TOPO vector (Fig.2.9.1.) has a single complimentary overhang, a 3' deoxythymidine (T) residue, allowing a ligation between the two products. This allows PCR inserts to ligate efficiently with the vector. Topoisomerase is attached to the vector, allowing the vector to be "activated", thus providing enough energy to spontaneously drive the reaction. The reaction takes only 5 minutes and longer incubation (30 minutes) could achieve maximum efficiency. pCR®4-TOPO vector also allows direct selection of recombinants via disruption of the lethal *E.coli* gene, *ccdB* which fused to the C-terminus of the LacZα fragment. Ligation of a PCR product disrupts expression of the lacZα- *ccdB* gene fusion permitting growth of only positive recombinants upon transformation in TOP10 cells. Cells that contain the non-recombinant vector are killed upon plating. Therefore, blue/white screening in other vectors such as pGEM-T is not required.

Fig. 2.9.1. Map of pCR®4-TOPO vector.



2.9.2. Ligation

Second round PCR products (1.0 µl) amplified by each primer pair were mixed with 1.0 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) provided in kit and sterile water to a final volume of 5.0 µl. After the addition of 1.0 µl pCR®4-TOPO vector, the mixture was incubated 30 min at room temperature. Longer incubation time will yield more colonies. The mixture was placed on ice before the transformation step.

2.9.3. *E.coli* transformation

The ligation mixture (2.0 µl) was added into a vial of One Shot® Chemically Competent *E.coli*, mixed gently, and left on ice for 20 min. After the heat-shock for 30 secs at 42°C water bath without shaking, the cells were immediately transferred onto ice. 250 µl of room temperature S.O.C. medium was added to the mixture. The tube was placed in 37°C shaking incubator to horizontally shake at 200rpm for 1 h.

2.9.4. Transformants sequencing and alignment

The transformation mixture (20 µl) was spread on a pre-warmed 100 µg/mL ampicillin selective Luria Broth (LB/Amp) plate and incubated overnight at 37°C. The white single colony was transferred into liquid LB/Amp culture and incubated overnight at 37 °C before direct colony sequencing by Plant Biotechnology Institute, National Research Council of Canada (Saskatoon). The software Chromas was used to read the chromatogram files. Sequences were aligned to previously published sequences of AMF species with the multiple sequence alignment program Clustal X.

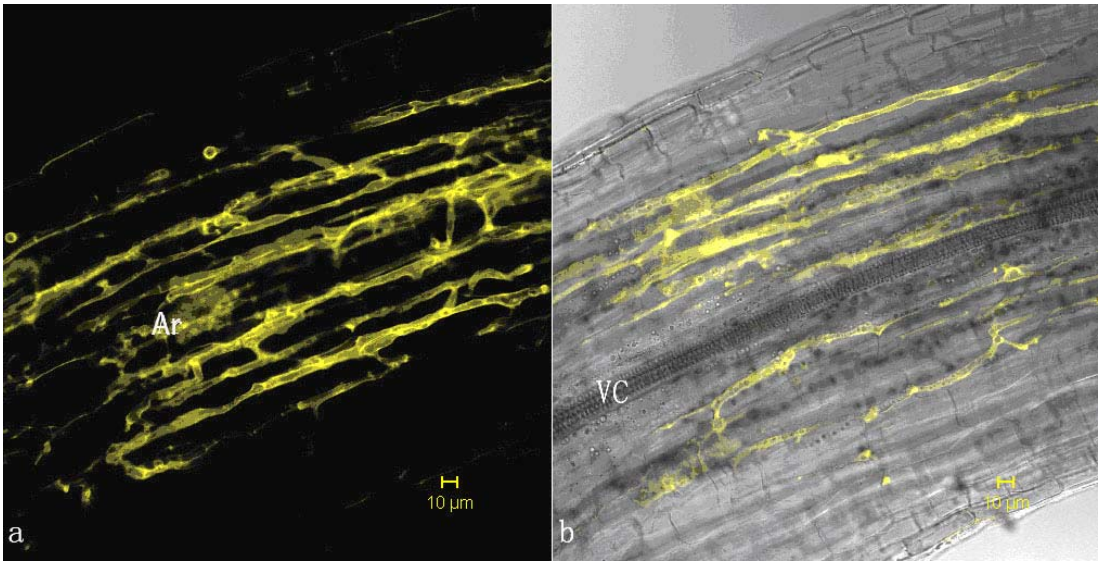
III. RESULTS

3.1. Endorhizal fungal morphology

3.1.1. Fungal hyphae

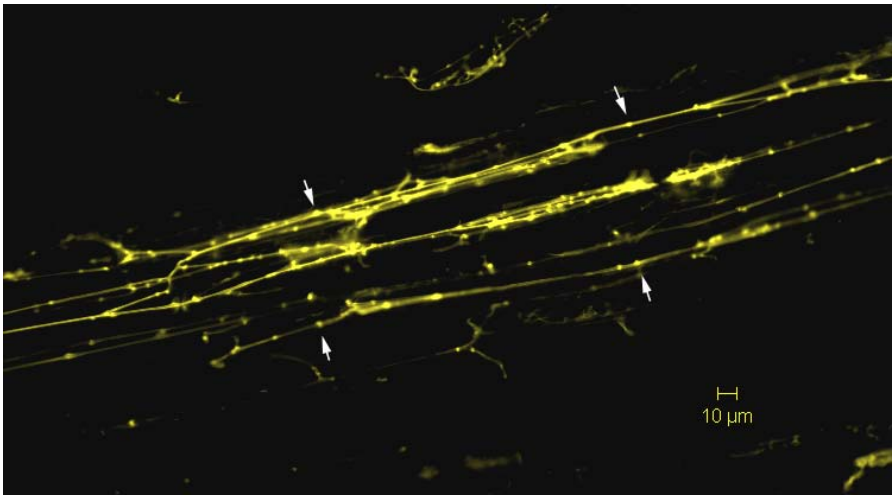
Different endorhizal fungi were observed colonizing the samples' roots. AMF, characterized as having 4-6 μm wide aseptate hyphae (Fig. 3.1.1.1) were the most prevalent fungi (Table 3.3). There was a low incidence of FE and SE in both roots of dandelion and chive.

Fig. 3.1.1.1. Paired confocal epifluorescence (a) and differential interference contrast (b) micrographs of lactofuchsin-stained AMF in dandelion lateral roots showing typical AM hyphae associated with arbuscules (Ar). The average diameter of aseptate hyphae was 4-6 μm and they grew intercellularly in the cortex. This growth resulted in a linear appearance, which was typical *Arum*-type AM. Figure (b) shows the location of the vascular cyclinder (VC). Scale bar = 10 μm.



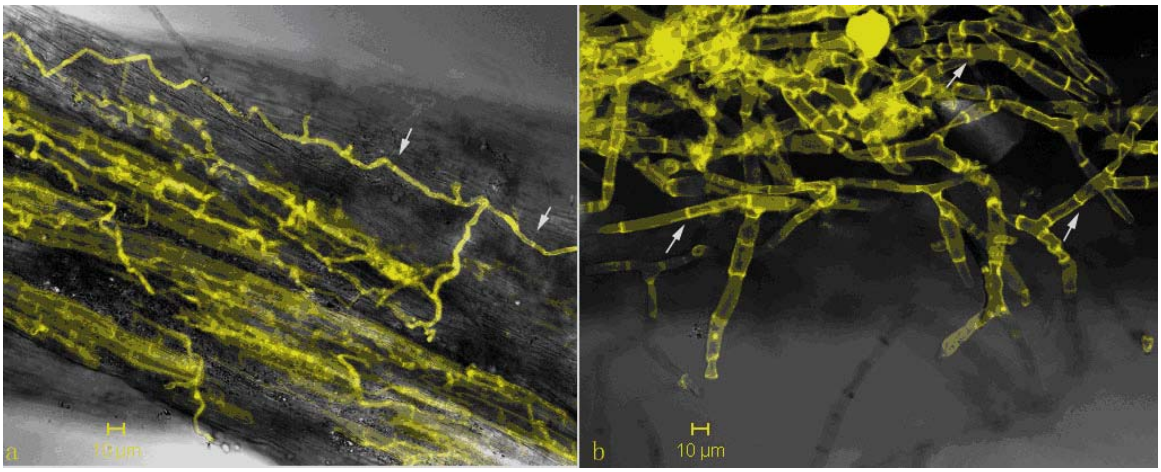
The hyphae of FE, 1- 1.5 µm wide, formed net-like hyphal structures with fungal swellings (bright dots) evenly distributing along the hyphae (Fig. 3.1.1.2). These swellings might be small vesicles protruding from FE, as reported in *Taraxacum hyparcticum* by Ormsby et al. (2007) and in *Trifolium subterraneum* by Thippayarugs et al. (1999).

Fig. 3.1.1.2. Fine endophytes (FE) in lactofuchsin-stained chive roots viewed with CLSM, showing net-like hyphal structure with fungal swellings, putative small vesicles (arrows). The diameter of hyphae ranged from 1.0 to 1.5 µm. Scale bar = 10 µm.



Septate hyphae (SE) were found both in the root cortex (Fig. 3.1.1.3, a) and on the surface (Fig. 3.1.1.3, b) of dandelion and chive roots. The occurrence of AMF, FE and SE in dandelion and chive indicated the diversity of endorhizal fungi colonizing the host plants.

Fig. 3.1.1.3. Septate endophytes (SE) in lactofuchsin-stained roots of dandelion and chive, viewed with CLSM. SE were found both in the root cortex of dandelion roots (a) and on the surface of chive roots (b), with evenly distributed septa (arrows) along the hyphae. Scale bar = 10 μm.



3.1.2. Arbuscules

AM fungal species varied in their arbuscular structures. The highly fine-branched arbuscules (Fig. 3.1.2.1) were associated with intercellular hyphae, but they did not have apparent trunk hyphae penetrating cortical cells. Fig. 3.1.2.2. showed tree-like arbuscules but of different patterns of their trunk hyphae. Some arbuscules had main trunk hyphae at the penetration site before branched to finer arbuscules (Fig. 3.1.2.2.c, arrowheads). Others had trunk hyphae branching to several lateral hyphae, from where fine arbuscules came out (Fig. 3.1.2.2. a, b, arrowheads). The diverse appearance of arbuscules might indicate that they were at different stages of development or they were of different fungal species, according to conventional identification methods based on morphological characteristics. FE were also found producing arbuscules (Fig. 3.1.2.3), which was also described by Allen et al. (2006).

Fig. 3.1.2.1. AMF in lactofuchsin-stained dandelion roots viewed with CLSM, showing extensive fine-branched arbuscules (Ar, arrowheads) associated with intercellular hyphae (IH, arrows) through the cortex. Scale bar = 10 μm.

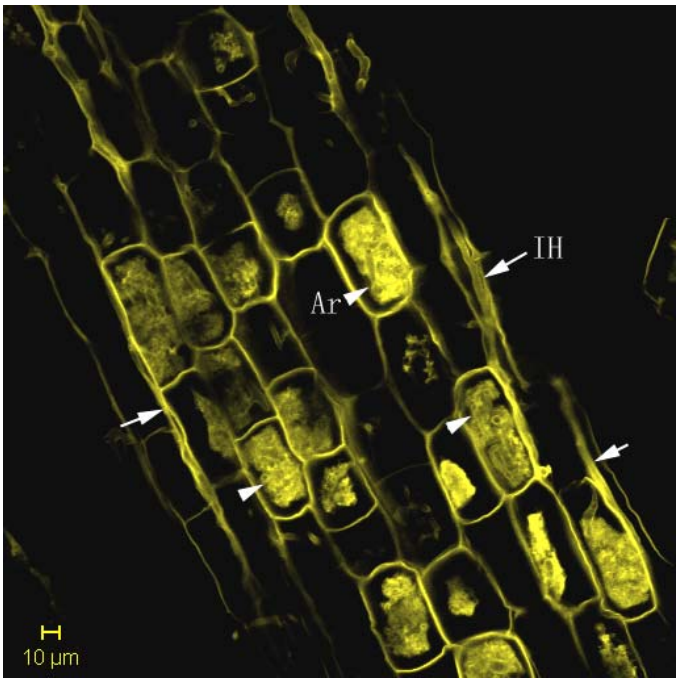


Fig. 3.1.2.2. Arbuscules with different patterns of trunk hyphae (arrowheads) in dandelion (a) and chive (b, c) root stained with lactofuchsin and viewed with CLSM. Scale bar = 10 µm.

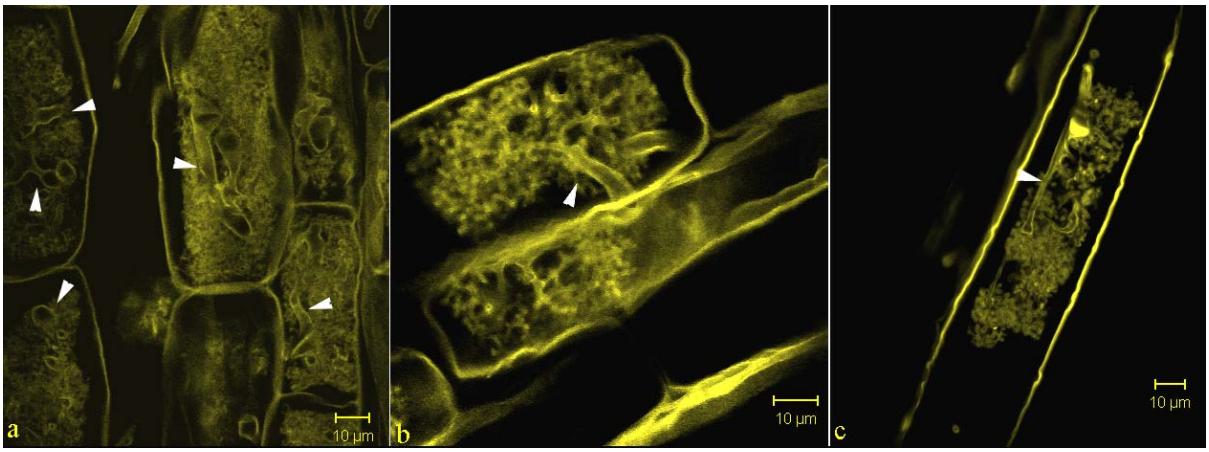
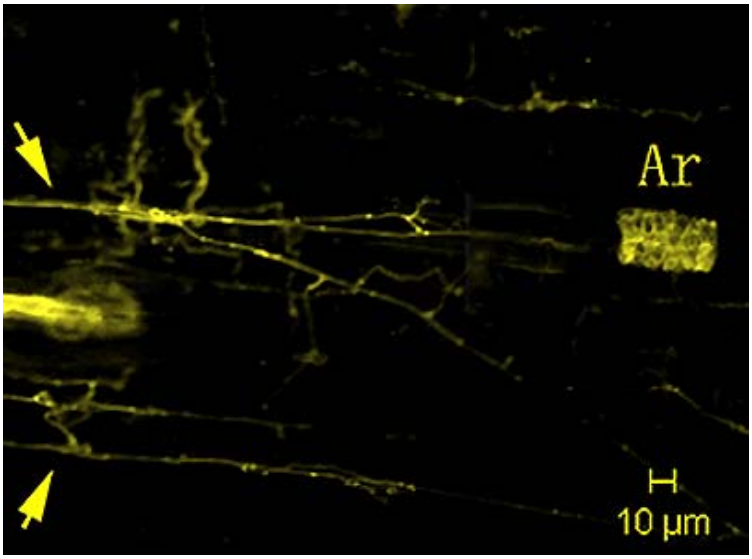


Fig. 3.1.2.3. FE in lactofuchsin-stained chive roots viewed with CLSM. Scale bar = 10 µm.



3.1.3. Vesicles

Vesicles were not commonly oberserved in dandelion and chive samples. Only samples collected around Oct 2006 had vesicles within roots, which accorded with the finding of Peterson et al. (2004) that vesicles form in roots towards the end of the growing season. The size of vesicles was relatively consistent, ranging from 20 μm to 30 μm in length.

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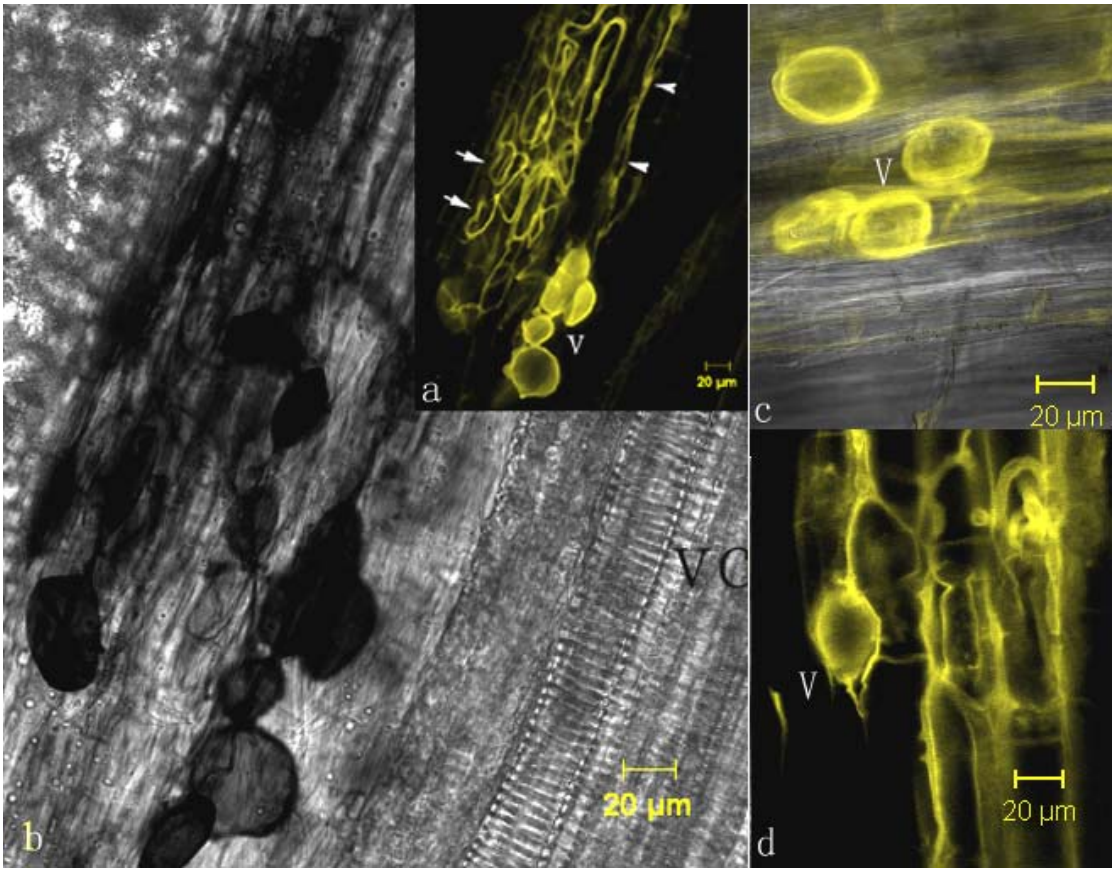
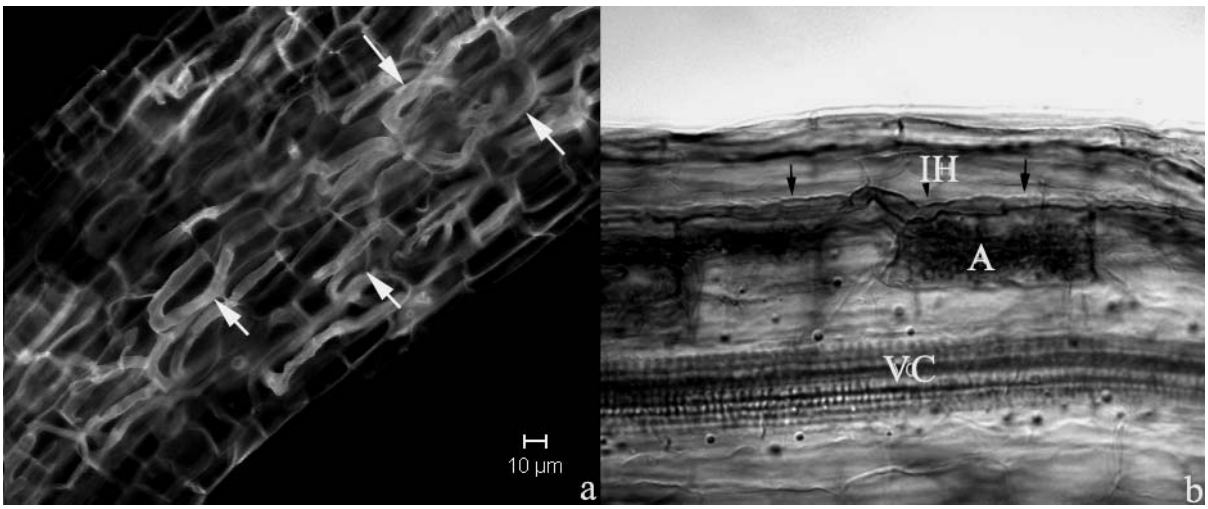
Fig. 3.1.3. Morphology of AM fungal vesicles (V) in lactofuchsin-stained dandelion (a, b,c) and chive (d) lateral roots viewed with CLSM. VC: vascular cylinder. Scale bar = 20 μm.



3.2. AM morphotypes in dandelion and chive roots

The diverse morphologies of hyphae, arbuscules and vesicles strongly suggested that the host plants were co-colonized by several endorhizal fungi. As a mutualistic group, AMF produced different morphotypes colonizing host roots. The typical AM morphotypes were observed in my root samples (Fig. 3.2.1). The appearance of hyphal coils and finely branched arbuscules wa in accord with the description of typical *Paris*- and *Arum*-type AM, respectively (Brundrett et al. 1985; Smith and Smith 1997).

Fig. 3.2.1. Typical *Arum*-and *Paris*-type AM structures. (a) Dandelion roots stained with LF and imaged with CLSM had typical *Paris*-type hyphal coils (arrows) in the cortex. (b) Chive roots stained with CBE and imaged with differential interference contrast image showed *Arum*-type intercellular hyphae (IH, arrows) associated with arbuscules (A) in cortex.



Besides the *Arum*- and *Paris*-types AM, the intermediate morphotype was also prevalent in both dandelion and chive roots: extensive intracellular hyphal coils (*Paris*-type AM) in the outer cortex and intercellular hyphae with arbuscules (*Arum*-type AM) closer to the stele of root (Figs. 3.2.2, 3.2.3). Allen et al. (2006) found similar fungal morphology in dandelions sampled from Arctic region.

Fig. 3.2.2. Image of intermediate AM morphology in the root of chive. Optical sections of dandelion root, 2 µm apart in depth (24, 26, 28, 30, 32 µm from the surface of root to the central vascular cylinder), stained with LF and imaged with CLSM (a-e) and the differential interference contrast image (f). (a-b) The intracellular hyphae were prevalent in the form of coils, which was characteristic for *Paris*-type AM. (c-e) *Arum*-type finely branched arbuscules were found close to the root vascular cylinder (h, VC) than hyphal coils. (e) The fine-branched arbuscules were derived from the hyphae. Scale bar = 10 µm.

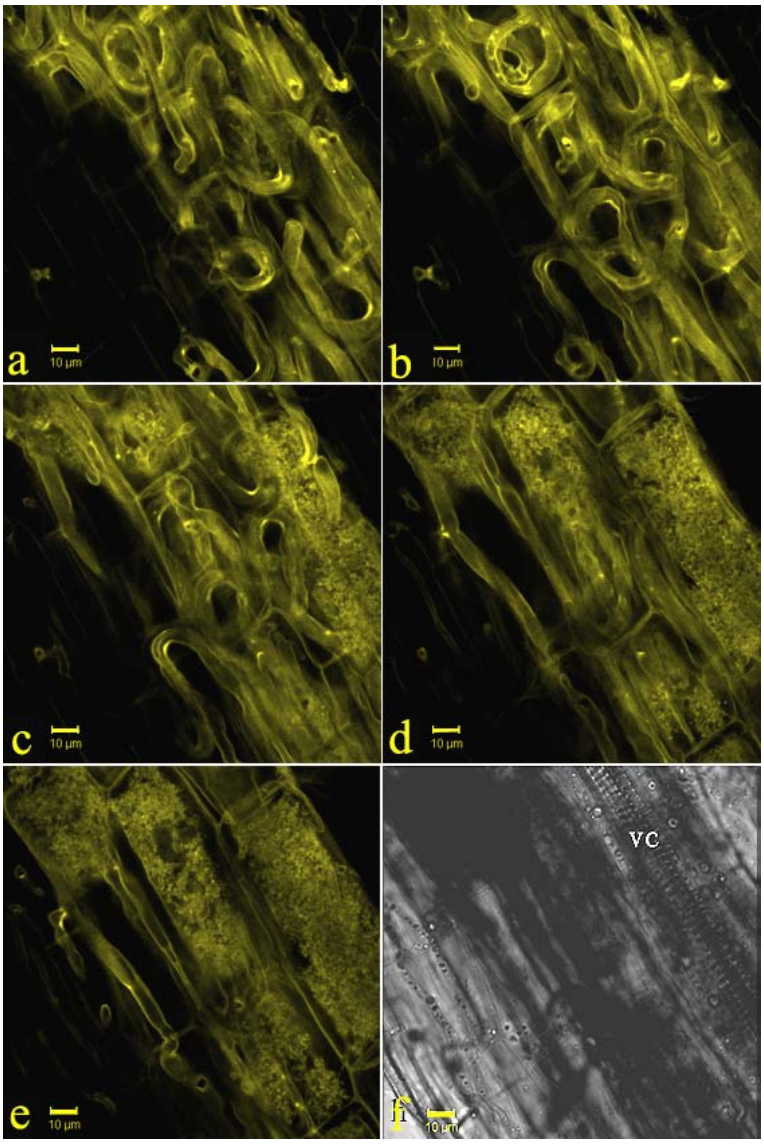
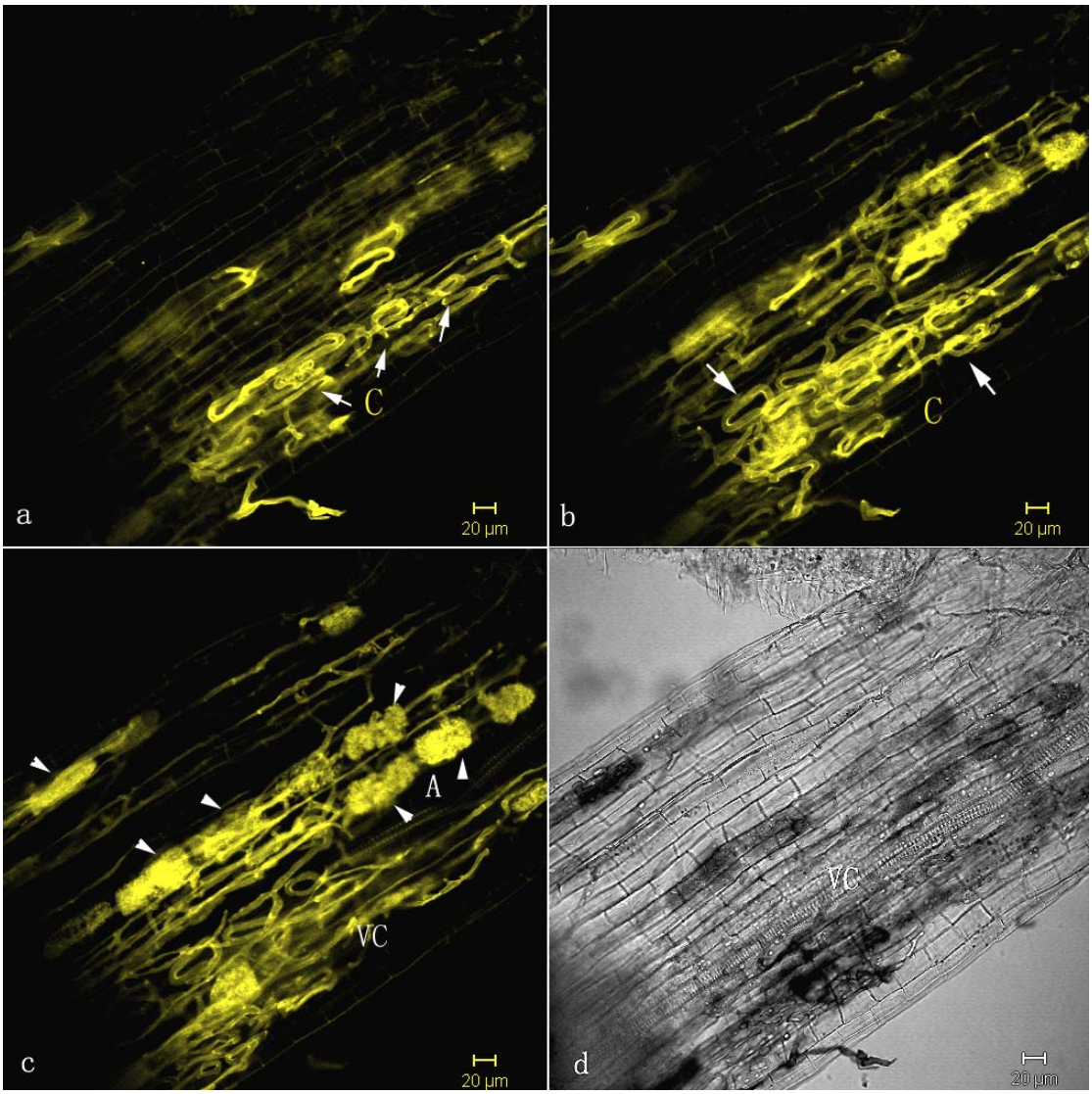


Fig. 3.2.3. Image of intermediate AM morphology in the root of dandelion. Optical sections, 7 µm apart in depth (30, 37, 44 µm from the root surface to the central vascular cylinder), of dandelion root stained with LF and imaged with CLSM (a-c) and the differential interference contrast image (d). (a-b) Extensive *Paris*-type hyphal coils (C). *Arum*-type finely branched arbuscules (arrowheads in c). The arbuscules were formed deeper in the root cortex, closer to the vascular cylinder (VC) than the hyphal coils. Scale bar = 20 µm.



3.3. Endorhizal fungal abundance

Quantitation of endorhizal fungi using MQM method was shown in Table 3.3. The results were based on individual root systems, each assessed at 150-200 intersections. The chive and dandelion hosted all three types of endorhizal interaction. This finding was consistent with reports of Ormsby et al. (2007) and Allen et al. (2006). However, there were not significant differences of abundance ($P > 0.05$ ANOVA) of hyphae of different endorhizal fungi between dandelion and chive, except the SE ($P < 0.05$, $F = 14.02$, ANOVA). In both dandelion and chive samples, the colonization of AMF was always much higher than FE and SE. In dandelion, there were significant differences of abundance between AM hyphae and SE hyphae ($P < 0.05$, $F=24.34$, ANOVA), AM hyphae and FE hyphae ($P < 0.05$, $F= 73.92$, ANOVA) and SE hyphae and FE hyphae ($P < 0.05$, $F= 7.74$, ANOVA). In chive, AM hyphae were always more abundant than SE ($P < 0.05$, $F= 23.82$, ANOVA) and FE hyphae ($P < 0.05$, $F= 24.28$, ANOVA). Furthermore, the data of intercellular hyphae and arbuscule and hyphal coils also indicated that mixed *Arum*- *Paris*- morphotypes were prevelant in both plant species. In each plant species, there was also variation of abundance of AM fungal structures, including intercellular hyphae, arbuscules and hyphal coils. In both dandelion and chive, the arbuscules was of lower abundance than that of intercellular hyphae and hyphal coils ($P < 0.05$ ANOVA).

Table 3.3. Endorhizal fungal colonization of dandelion and chive root systems.

Plant	N of plants	AM hyphae	Intercellular hyphae	Arbuscules	Hyphal coils	SE hyphae	FE hyphae
Dandelion	4	63.2 ± 7.5 a	58.6 ± 4.9 [1]	30.2 ± 3.9 [2]	49.3 ± 5.7 [1]	32.5 ± 7.7 b	8.9 ± 4.8 c
Chive	4	52.1 ± 9.9 a	42.3 ± 4.4 [3]	16.8 ± 4.6 [4]	33.9 ± 5.0 [3]	7.5 ± 2.7 c	8.1 ± 2.6 c

The root samples were examined microscopically (400 x objective) using the MQM method. Data were expressed as the mean ± standard error of the mean. Samples are listed in Table 2.2. The data followed by a different letter or number in column and row were significantly different ($P < 0.05$ ANOVA).

3.4. Soil properties

The pH of garden soil (pH 6.7) was lower than roadside soil (pH 7.2) sampled in this study. There were significant differences in extractable-P and organic matter between the two sites (Table 3.4, $P<0.01$ ANOVA), and both soils were fertile with high content of P and organic matter. The fact that garden soil with lower pH had higher P content accords with the finding of Hamel (2004) that decreased pH increases the solubility of P precipitates. In our study, there were no significant differences of AMF abundance of dandelions collected from two soils (Table 2.2).

Table 3.4. pH, organic matter, extractable-P of garden and roadside soils from where dandelion roots were sampled as well as the AM fungal abundance.

Soil	N of plants	pH	Organic matter (%)	Extractable-P (ppm)	AMF abundance (%)
Garden	2	6.7	6.9 ± 0.1 a	84.8 ± 0.3 a	61.0 ± 4.6 a
Roadside	2	7.2	4.0 ± 0.1 b	45.6 ± 0.3 b	65.4 ± 15.4 a

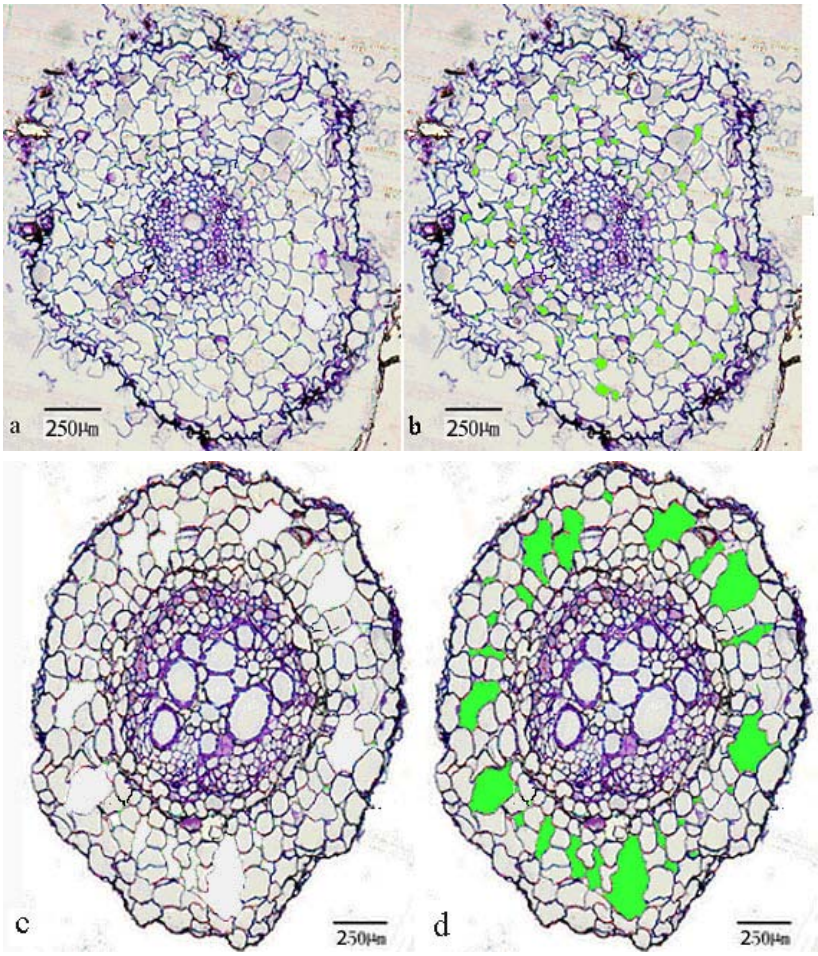
The root samples were examined microscopically (400 x objective) using the modified intersection method (see Materials and Methods). Data are expressed as mean ± standard error of the mean. Plant samples were listed in Table 2.2. Means in a column followed by a different letter were significantly different ($P< 0.01$ ANOVA).

3.5. Root cross-sections

3.5.1. Root cell packing of dandelion

Dandelions have a taproot system. The diameter of lateral roots ranges from 1.25 mm to 1.50 mm. Depend on the age of lateral roots of dandelion, they displayed different root cell packing patterns. Young roots had small percentage of xylem (7.52 %), small stele (300-350 μm) and compact cortical cells (Fig. 3.5.1.a). Older roots had large percentage xylem (26.7 %), larger stele (600-850 μm) and loosely packed cortical cells (Fig. 3.5.1.b).

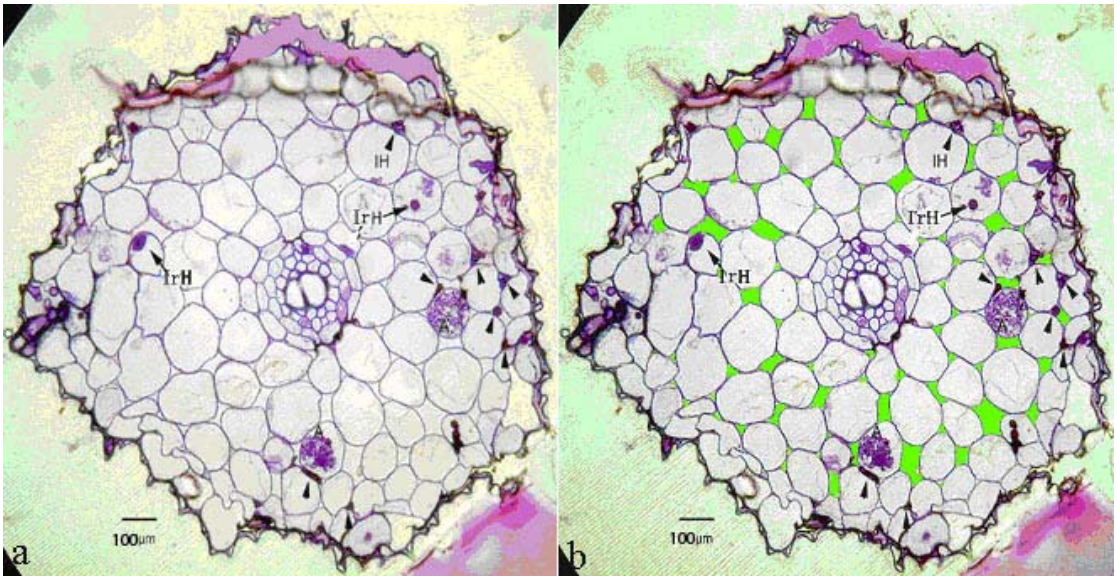
Fig.3.5.1. Toluidine-stained semi-thin cross sections (0.9 μm thick) of dandelion roots. The second copy figures (b, d) marked the air space (AS) as green color. (a, b) Root with less xylem and relatively compact cell packing. (c, d) Root with large percentage xylem and loosely packed cortical cells. Scale bar = 250 μm.



3.5.2. Root cell packing of chive

Chive had a fibrous root system, and its cell-packing pattern was relatively more consistent than dandelion lateral root. Typically, chive roots had a small percentage of xylem and loosely packed cortical cells. There was typical *Arum*-type AM colonizing the cortical cells. The diameter of chive roots ranged from 1.0 mm to 1.2 mm. Some semi-thin cross sections contained AMF, showing arbuscules (A) produced from the adjacent intercellular hyphae. There were also intracellular hyphae visible but of low quantity.

Fig.3.5.2. Toluidine-stained semi-thin cross section (0.9 μm thick) of chive roots. The second figure marked the intercellular air space (AS) as green. *Arum*-type arbuscules (A) produced from the adjacent intercellular hyphae (IH, arrowheads), filled up some cortical cells. IH were mainly found in the outer layers where the cells were loosely packed. Intracellular hyphae (IrH, arrows) were also found in the cortex. Bar = 100 μm.



3.5.3. Air spaces (AS) of host roots

In this study, I investigated the relationship between plant root cell packing and AM morphology. Brundrett and Kendrick (1988, 1990b) and Smith and Smith (1997) suggested that the distribution of AS in root cortex could influence AM morphotype. In my study, the hyphal colonization with respect to intercellular hyphae, arbuscules and hyphal coils of roots of dandelion was much higher than those of chive (Table 3.3, $P < 0.05$ ANOVA). However, there was no significant difference of the proportion of intercellular AS, both inner and outer cortex, between the two plant species (Table 3.5.3. $P > 0.05$, ANOVA). Therefore, higher abundance of intercellular hyphae (*Arum*-type AM) in dandelion roots did not correlate with the bigger intercellular AS in cortex, nor did higher abundance of arbuscules with the smaller AS in inner cortex. Also the lower abundance of hyphal coils (*Paris*-type AM) in the roots of chive did not necessarily mean the smaller percentage of intercellular AS in outer cortex. Furthermore, in the dandelion and chive, there was no significant difference between inner, outer and total AS, even though the abundance data of fungal structure (Table 3.3) varied substantially. The occurrence of high abundance of intercellular hyphae (*Arum*-type) and hyphal coils (*Paris*-type) did not support the model proposed by Brundrett and Kendrick, that larger outer AS facilitated the growth of intercellular hyphae but not hyphal coils. In summary, there was no apparent correlation of plant root cell packing with AM morphology in my study.

Table 3.5.3. The proportion of air space of total cross-section, inner and outer cortex in the roots of dandelion and chive.

Plant	N of plants	N of cross sections	Inner AS	Outer AS	Total AS
Dandelion	4	8	7.2 ± 1.1 a	11.7 ± 2.6 a	8.3 ± 1.5 a
Chive	4	8	4.8 ± 1.0 a	5.1 ± 1.1 a	4.7 ± 1.0 a
F-value			6.70	17.70	10.30
P-value			> 0.10	> 0.05	> 0.05

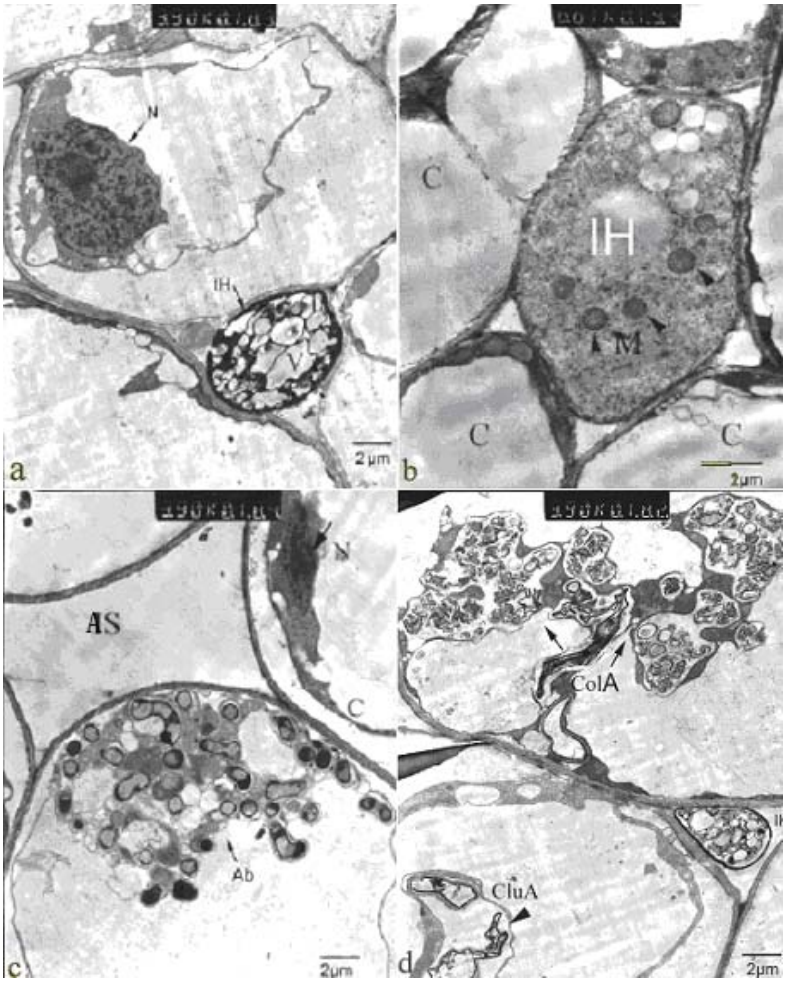
Plant samples were listed in Table 2.2. For each plant sample, two cross sections were processed with calculation of proportion of AS. Totally eight cross sections were analysed for each plant species. Repeated measurement was used to support the validity of statistics, considering the small sample size. Data were expressed as mean ± standard error of the mean.

3.6. AMF ultrastructure

In dandelion roots, uninfected cortical cells were highly vacuolated and few organelles were observed (Fig. 3.6: b, c cortical cells). The contents of hyphae appeared denser than the plant cells (Fig. 3.6: a, vacuole; b, mitochondria). The circular profiles of intercellular hyphae (Fig. 3.6: a, b, d) were generally 4-7 μm in diameter, leaving space surrounding them in triangular profiles.

Arbuscules at different developmental stages previously described by many researchers (Kinden and Brown 1975; Yawney and Schultz 1990) were present in the roots. Young arbuscules (Fig. 3.6: c) were densely cytoplasmic and had numerous, distinct branches ranging from 0.5 to 2.0 μm in diameter and usually occupied a large proportion of the cross section of a cortical cell. The cytoplasm of arbuscules became progressively disorganized as they aged, described here as collapsing arbuscules (Fig. 3.6: d): distinct branches together with combined branches had turned into loosely connected strands. The profile decreased in area and distinct branches were less evident, described here as the clumped stage of arbuscules (Fig. 3.6: d). Clumped arbuscules were centrally located in the host cell and occupied relatively little of the plant cell root profile.

Fig. 3.6. TEM images of AMF colonizing dandelion lateral roots. a: Intercellular hypha (IH) contain dense cytoplasm and abundant vacuoles (V). Uninfected cortical cells were highly vacuolated and the nucleus (N) was normally positioned in the periphery of cell. b: High magnification of IH showing dense cytoplasm and abundant fungal mitochondria (M, arrowheads). c: Young arbuscules (Ab) in a cortical cell have smooth profiles and dense contents. d: Collapsed arbuscules (Col A) and Clumped arbuscules (Clu A) have disorganized contents. Scale Bar = 2 µm.



3.7. Nested PCR products

Dandelion 1-4 and Chive 3 samples (Table 2.2) harvested in Saskatoon were extracted for DNA, which was used as template for nested PCR as described in Materials and Methods 2.7, 2.8. The first round PCR used the universal primers NS5 and ITS4 to generate a PCR product of approximately 1200 bp (Fig. 3.7.1). The PCR products of the second round PCR showed various banding patterns (Figs. 3.7.2, 3.7.3, 3.7.4, 3.7.5, 3.7.6). The size of each PCR product amplified from nested PCR was listed in Table 3.7.

As shown in Table 3.7, some samples had more than one single band following second round PCR with one pair of primers. Others did not produce a second round product (non-amplicon, NA), which might be due to the absence of target AMF groups or the failure of nested PCR. If the PCR product was similar to the expected size, it indicated the possible detection of AMF in the primer target groups. The bands of PCR products of dandelion 1 did not fit any expected size (Fig. 3.7.2). Dandelion 2 only shared one similar size (660 bp, arrowhead in Fig. 3.7.3) with primer ACAU1661– ITS4i (645 bp), which was possibly colonized by fungi of Acaulosporaceae group. In dandelion 3, the 680 bp PCR product (arrowhead in Fig. 3.7.4) amplified with LETC1670– ITS4i had similar size as expected 676 bp, of *Glomus etunicatum / claroideum* group. The PCR products of dandelion 4 had three bands of expected sizes (arrowheads in Fig. 3.7.5): 650 bp PCR product with ACAU1661– ITS4i (645 bp), 680 bp PCR product with LETC1670– ITS4i (676 bp) and 300 bp PCR product with GIGA5.8R– ITS1F (305 bp). Dandelion 4 may be colonized by fungi of *Glomus etunicatum/claroideum* group, Acaulosporaceae and Gigasporaceae. Chive 3 had two PCR products (1080 bp, 650 bp, arrowhead in Fig. 3.7.6) around the expected size with primers ARCH1311– ITS4i (1052 bp) and LETC1670– ITS4i (676 bp), indicating the possible fungi of *Archaeospora gerdemannii/ trappei* group, *Glomus occultum*/ *brasilianum* group and *Glomus etunicatum / claroideum* group in roots. The successful amplification of bands of

expected size is only the first stage of the molecular identification process. Further cloning and sequence work is required to verify the fungal species within root samples.

Fig. 3.7.1. Agarose gel showing first round PCR products (around 1200 bp) of dandelions (D1, D2, D3, D4) and chive (C) with universal primers NS5-ITS4. Lane N: negative control. 100bp fragment ladder (Invitrogen, Canada) with 1000bp fragment marked.

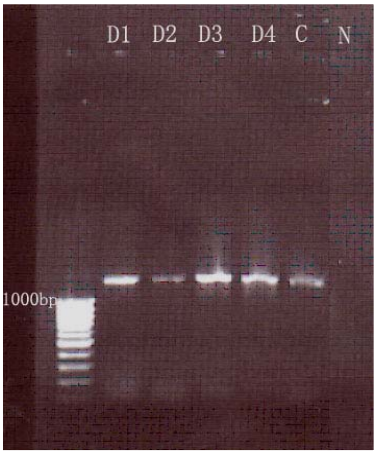


Fig. 3.7.2. Agarose gel showing rRNA-ITS gene fragment amplified from extracted genomic DNA of dandelion 1 (D1) from garden soil. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F. 100bp fragment ladder (Invitrogen, Canada) with 1000bp, 500bp fragments marked. The bands of PCR products of D1 did not fit any expected size.

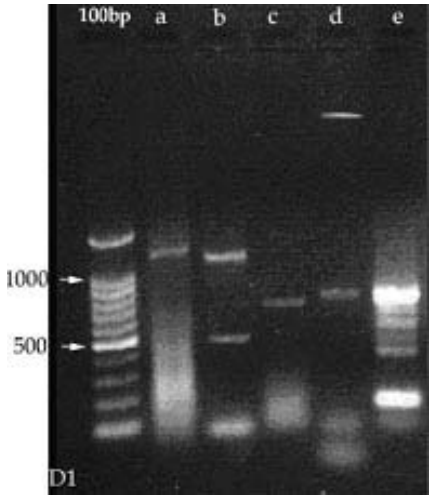


Fig. 3.7.3. Agarose gel showing rRNA-ITS gene fragment amplified from extracted genomic DNA of dandelion 2 (D2) from garden soil. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F. 100bp fragment ladder (Invitrogen, Canada) with 1000bp, 500bp fragments marked. D2 only shared one similar size (660 bp, arrowhead) with primer ACAU1661– ITS4i (645 bp), which was possibly colonized by fungi of Acaulosporaceae group.

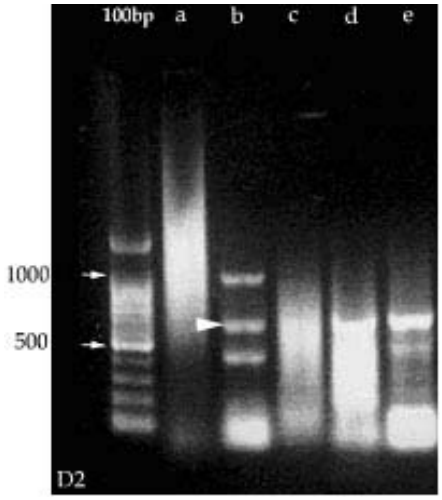


Fig. 3.7.4. Agarose gel showing rRNA-ITS gene fragment amplified from extracted genomic DNA of dandelion 3 (D3) from roadside soil. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F. 100bp fragment ladder (Invitrogen, Canada) with 1000bp, 500bp fragments marked. In D3, the 680 bp PCR product (arrowhead) amplified with LETC1670– ITS4i had similar size as expected 676 bp, of *Glomus etunicatum / claroideum* group.

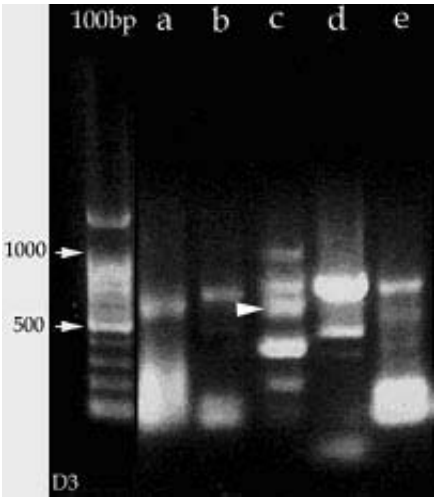


Fig. 3.7.5. Agarose gel showing rRNA-ITS gene fragment amplified from extracted genomic DNA of dandelion 4 (D4) from roadside soil. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F. 100bp fragment ladder (Invitrogen, Canada) with 1000bp, 500bp fragments marked. The PCR products of D4 had three bands of expected sizes (arrowheads): 650 bp PCR product with ACAU1661– ITS4i (645 bp), 680 bp PCR product with LETC1670– ITS4i (676 bp) and 300 bp PCR product with GIGA5.8R– ITS1F (305 bp). D4 may be colonized by fungi of *Glomus etunicatum/claroideum* group, Acaulosporaceae and Gigasporaceae.

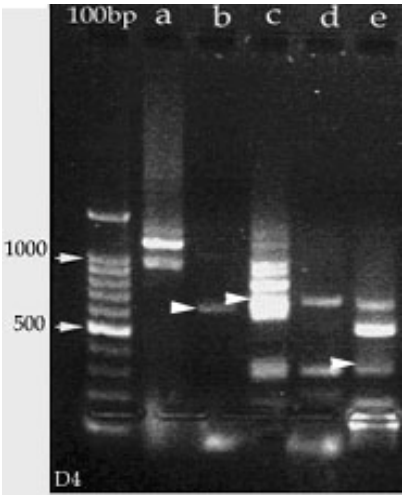


Fig. 3.7.6. Agarose gel showing rRNA-ITS gene fragment amplified from extracted genomic DNA of chive (C) from garden soil. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F. 100bp fragment ladder (Invitrogen, Canada) with 1000bp, 500bp fragments marked. Chive had two PCR products (1080 bp, 650 bp, arrowhead) around the expected size with primers ARCH1311– ITS4i (1052 bp) and LETC1670– ITS4i (676 bp), indicating the possible fungi of *Archaeospora gerdemannii/ trappei* group, *Glomus occultum*/ *brasilianum* group and *Glomus etunicatum / claroideum* group in roots.

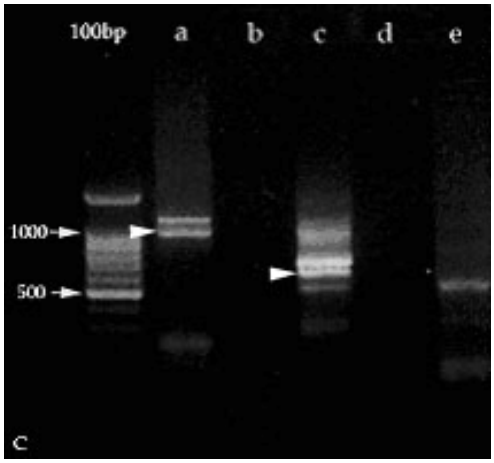


Table 3.7. Amplified product sizes of dandelions 1, 2 (D1, D2) sampled from garden soil, dandelions 3, 4 (D3, D4) from roadside soil, chive 3 (C) from garden soil, in comparison with expected size of each primer pair. Lane a: ARCH1311– ITS4i; Lane b: ACAU1661– ITS4i; Lane c: LETC1670– ITS4i; Lane d: GLOM1310– ITS4i; Lane e: GIGA5.8R– ITS1F.

Lane	a: Archaeospora gerdemannii/ trappei Glomus occultum/ brasilianum	b: Acaulosporaceae	c: Glomus etunicatum/ claroideum	d: Glomus mosseae/ intraradices	e: Gigasporacea
Expected size (bp)	1052	645	676	1012	305
D1 (bp)	1300	1200,520	750	850	850,700,600, 500,450,200
D2 (bp)	NA	1000,660,450	NA	NA	700,500
D3 (bp)	650	750	1100,800, 680, 350,200	800,450	800,200
D4 (bp)	1200,980	650	950,900,800, 680,500,300	680,300	650,500,300
C (bp)	1200,1080,200	NA	750,650, 550,300	NA	570

3.8. PCR products cloning

The second round PCR products of each sample were cloned into pCR®4-TOPO vector and transformed into *E.coli*, followed by the direct sequencing of 10 colonies of each PCR products. Most sequencing failed to obtain a noise-free signal, which impedes the fungal identification.

IV. DISCUSSION

4.1. Diversity of endorhizal fungi

Endorhizal fungus—plant root interactions in herbaceous plants include AMF, SE and FE (Peterson et al. 2004). There are limited studies reporting the occurrence of multiple types of endorhizal fungi within one plant (Rincón et al. 1993; Allen et al. 1998; Smith et al. 2003; Wubet et al. 2003; Pattinson et al. 2004; Fisher and Jayachandran 2005; Leung et al. 2006). Many studies focus on the physiology of mutualistic AMF, so there is relatively less knowledge of SE and FE. In addition, conventional transmitted light microscopic technique has not routinely detected the fine structures of FE. Olssen et al. (2004) and Ormsby et al. (2007) demonstrated the prevalence of AM and FE fungi in host roots. Ormsby et al. (2007) mentioned the appearance of SE in host roots in addition to other types of endorhizal fungi.

In my study, dandelions and chives were harvested from undisturbed roadside, lawn, and garden soil in Saskatoon (SK, Canada). When examined with epifluorescence microscopy following LF staining, these plant roots were found to be colonized by several types of endorhizal fungi. In order to assess the abundance of each type of endorhizal fungus, I used a multiple quantitation method (MQM). In both dandelions and chives: 1) SE were distinguished for their relatively evenly spaced septa; 2) FE hyphae were of narrow diameter (1-2 μm); 3) AMF were characterized for 5-6 μm wide aseptate hyphae, arbuscules and vesicles. This study showed that plant roots can simultaneously associate with more than one type of endorhizal fungi. Besides the morphological differences between endorhizal fungi, there were also varied fungal structures within certain fungi, such as arbuscules. As for morphological diversity, Merryweather and Fitter (1998) showed that different AMF could simultaneously colonize a single root segment. It remained unclear this diversity was due to multiple fungal species or of their own diverse characters. The molecular identification

technique could be used to clarify the species colonizing host roots. Studies regarding endorhizal fungus type and abundance in plant roots are indispensable to explore their distribution and influence in ecosystems.

4.2. Factors affecting endorhizal fungal abundance

Diverse factors, such as host-fungus compatibility, multiple fungal species, soil properties, temperature, season and their potential combinations, complicate the understanding of the variation of fungal abundance in plant roots. My study examined the influence of soil properties, including soil P status, organic matter and pH, to endorhizal fungal abundance. Low soil P concentrations have been reported to help increase hyphal growth (Nagahashi et al. 1996; Douds and Nagahashi 2000). In my study, at two soil sites, there were no significant differences of AMF colonization in soils of different P content. Nogueira and Cardoso (2006, 2007) reported that there was a range of P availability in which plant response to mycorrhiza was positive. The intermediate P level (50- 100 ppm) did not affect the growth of plant. In the case of enough available P, plants usually do not respond to mycorrhiza. If the P levels of garden and roadside soils are within this range, it is not expected to find differences of fungal abundance in dandelions. This preliminary study only examined soils from two different sites, where the P contents of soil were both over 45 ppm. It was not convincing enough to draw conclusion of P influence to fungal abundance.

Soil organic matter, the carbon compounds from decomposing organic materials, can be used as an index of the soil fertility (Paul and Clark 1989). Accumulation of organic matter seems to be important to provide a substrate where some kind of mycorrhizal association could develop (Allen et al. 1993). However in my study, different organic matter content in two soils did not correlate with variation in mycorrhizal abundance. Future study to investigate a larger sample size will give a more conclusive answer to the relationship between soil organic matter and fungal abundance.

Soil pH can induce the preference of certain fungi to colonize plant roots (Porter et al. 1987a, b). *Acaulospora leavis* is generally found more in acid (pH 4.5-4.9) than in neutral soil and germination of spores was limited at pH> 6, whereas three different strains of *Glomus* adapted better to soils ranging from pH 5.5-8.4 (Porter et al. 1987a, b). Therefore, the fungus adapted in certain soil will have greater chance to heavily colonize host plants. In my study, there was no apparent difference of fungal abundance. But we also cannot exclude the possibility that some AMF are less affected by soil pH. As Abbott and Robson (1985) reported, an isolate of *Glomus invermaium* colonizing roots of subterranean clover could survive in soil with pH ranging from 5.3 to 7.5. Therefore, the influence of soil pH to fungal abundance might be too minor to be observed.

In summary, whether soil environment is a key factor influencing AM fungal abundance requires more study as least for the plants of dandelion and chive examined in our study. Other mechanisms could have contributed to the abundance of mycorrhizae, including hormonal effects of the host plant or other microorganisms in the mycorrhizosphere (Barea et al. 2002). But the study of soil influence to endorhizal fungi is indispensable as the agricultural application is mainly focused on the improvement of soil fertility. Furthermore, considering that only four dandelion samples were processed, it might be due to insufficient data to see a difference.

4.3. Complexity of AM morphotypes

Most morphological studies (Smith and Smith 1997; Cavagnaro et al. 2001b; Koske and Gemma 2002; Yamato 2004; Dickson 2004; Fisher and Jayachandran 2005; Ahulu et al. 2006; Allen et al. 2006) focused on the morphological description of *Arum- Paris*-type AM. It used to be generally believed that most structures could be easily defined (Gallaud 1905). Therefore, other structural types were overlooked due to the ignorance of their significance or the fact that they did not fit into the definition

of AM symbioses (Widden 1996). Recently, accompanied by improved microscopic technique (Merryweather and Fitter 1991; Allen et al. 2006), more and more studies showed that identification of fungal structures and their morphotypes is not easily defined. Dickson (2004) concluded that intermediate structures were clearly present and that there was a continuum of structures. In our study, we also found intermediate AM morphotypes prevalent in host roots of both species. This intermediate morphotype is not equal to the description of Smith and Smith (1997), a mixture of the *Arum*- and *Paris*-type AM, but more in accordance with the classification of Dickson (2004) that "structures in between *Arum*- and *Paris*- morphologies". I think it is appropriate to interpret intermediate morphotypes as a transition between two extremes *Arum*- and *Paris* morphotypes. The explanation for this common intermediate morphotype needs further studies of fungal diversity of plants in the field. It is possible that roots can be colonized by more than one AMF, which is normal in the field as reported by Yamato (2004), and different AMF species may display characteristic *Arum*- or *Paris*- morphotype. Or rather the AMF is capable of displaying a transition of two morphotypes once associated with certain host plant.

As Dickson et al. (2007) reported, the genera of Alliaceae and Asteraceae predominantly formed *Arum*-type AM. The dandelion (Asteraceae) and chive (Alliaceae) samples in my study harbored both typical *Arum*- *Paris*- and transition morphotypes, indicating the complexity of AM morphotypes. Although there are 'robust' *Arum*- or *Paris*-type forming plants apparently independent of the influence of AMF colonizing them (Smith and Smith 1994; Dickson et al. 2007), various factors, possibly host plant, AMF and the environment, may work together to induce certain AM morphotypes.

4.4. The quantitative relationship between AM morphotype and host root anatomy

This is the first report to quantitively study the influence of root anatomy to the

formation of AM morphotype. Other researches suggested that larger AS in the root cortex facilitated the rapid colonization of intercellular hyphae (Brundrett et al. 1985) and high level of hyphal coils resulted from small intercellular AS present (Smith and Smith 1997). However, in this study, although the abundance of intercellular hyphae and hyphal coils were of significant differences between chive and dandelion roots, there was no difference of AS percentage regardless of location within the cortex. In the roots of both dandelion and chive, the abundance of intercellular hyphae and hyphal coils was similar, which does not support the assumption that intercellular hyphae grew preferentially in roots with large AS, whereas hyphal coils in roots lack of AS. Also there were no statistical data to support the assumption of previous studies. However, there are potential problems of my study: 1) the negative result might be due to the low number of semi-thin cross-sections investigated; 2) cross sections investigated do not always contain hyphal structures, which might reduce the fidelity of translating the effect of root anatomy to fungal formation if without fungus present. Therefore, future studies with high-resolution microscopy to easily present root cell packing are worthwhile to testify its influence to AM morphotypes. Furthermore, field materials are important to reveal the morphologies of endorhizal fungi in various ecosystems. But the field samples usually have more complex fungal communities, including AMF, SE and FE, which will complicate the analysis of the influence of the root cell packing to the AM morphotypes. A clean culture system, such as a root organ culture or an axenic-root-system plant culture, can control AMF within plant roots by inoculating a known glomalean species. It will be easier to study the symbiosis of certain AMF species and plants, further to understand the role of root cell packing to AM morphotypes.

4.5. Molecular analysis of AMF diversity in host plants

My study used molecular methods to assess AMF diversity in the plant roots of dandelion and chive. The nested PCR products display multiple banding patterns, not

the single band as described by Redecker (2000). As reported by Shepherd et al. (2007) who employed group-specific PCR to identify AMF in the roots of young trees from a field site in southern Costa Rica, the unexpected band was be genetrated by non-target organism, such as Ascomycete. Furthermore, Shepherd et al. (2007) reported more band sizes different from the study of Redecker (2000) and verified their AMF groups via sequencing. The non-specificity of the PCR primers may cause the amplification of unexpected bands. Further work to clone and sequence the PCR products is required to confirm if they are of AMF species or other fungi.

Each individual plant, even the same species found in the same soil, does not always have the same amplified PCR products. Two dandelions (D1, D2) from garden soil are collected at the same time and the same place. They only share two bands of same size (700, 500 bp) with primer GIGA5.8R– ITS1F among 16 PCR products. The rest of PCR products amplified with other primer pairs are all of different sizes. The same phenomenon is found in two dandelions (D3, D4) collected from same roadside soil. They only have one same band (800 bp) with primer LETC1670– ITS4i among 25 PCR products. The inconsistency of PCR banding pattern in plant samples collected from the same location complicate the fungal identification.

Vandenkoornhuyse et al. (2002) reported the high fungal diversity in grass *Arrhenatherum elatius*, using univseral primers to identify all fungi in roots. The diversity from the roots of a single plant species and from a single sampling location was completely unexpected. The 49 phylotypes were distributed across all fungal phyla: Chytndiomycota, Zygomycota, Basidiomycota, Ascomycota and Glomeromycota. In addition to the unexpected width of taxa, their study suggested the existence of unknown groups of fungi. Therefore, in my study with field collected samples, if the primers are not specific enough to target AMF which was recently reported by Shepherd et al. (2007), it is not surprising to have multiple PCR products, indicating the presence of multiple fungi. And the cloning and sequencing work did not provide informative results to identify AMF within plant roots.

The diversity of fungal communities in plant roots is much more complicated than previously thought. However, molecular techniques focus on distinguishing different AMF in host roots, but not address abundance. And fungus of certain abundance will have ecological implication to the host plant. The MQM is inexpensive, reproducible, and could capture information on colonization by multiple endorhizal morphotypes. Therefore, the combination of molecular technique with the fungal abundance investigation in host roots will provide more constructive information. In summary, this study is not conclusive enough to distinguish the AMF species in dandelion and chive samples. Further work to separately clone and sequence single PCR product is required to clarify the mycorrhizal groups in host plants. A larger sample size than in the current study is also indispensible to increase the power to detect differences of mycorrhizal diversity between plant species and the potential influence of soil types.

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